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(54) Title: CONJUGATES OF GONADOTROPIN RELEASING HORMONE

(57) Abstract

The present invention provides conjugates of GnRH and an immunogenic carrier protein wherein said GnRH is first coupled to a lysine-containing hydrophilic linear oligopeptide scaffold. The conjugates of the invention are capable of eliciting strong immune response against GnRH, and therefore are useful as immunosterilants for animals, or for therapy in animals and human for steroid hormone dependent tumors, and other conditions such as endometriosis and precocious puberty.

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TITLE OF THE INVENTION

# CONJUGATES OF GONADOTROPIN RELEASING HORMONE

## CROSS REFERENCE TO RELATED APPLICATIONS

This application is based on, and claims priority from, provisional application number 60/005,905 filed October 27, 1995.

## BACKGROUND OF THE INVENTION

The present invention relates to conjugates comprising one or more gonadotropin releasing hormone (GnRH) moieties linked to an immunogenic carrier protein via an oligopeptide scaffold. The conjugates of the present invention are effective immunosterilants, and they may also be used to arrest development of steroid hormone stimulated tumors.

Considerable interest exists with respect to the subject of sterilization of animals. This is especially true of those concerned with veterinary medicine and animal husbandry, particularly as they relate to the subject of sterilization of domestic animals such as dogs, cats, cattle, sheep, horses, pigs, and the like. Sterilization may be used to control undesirable gonadal steroid hormone driven behavior such as aggression in males and estrus behavior in females, to improve carcass quality in food animals such as swine and cattle, and to eliminate boar taint in the carcasses of male pigs.

Various methods have been developed over the years to accomplish sterilization. For example, with respect to male cattle, the most widely used procedure for eliminating problems of sexual or aggressive behavior is sterilization through surgical castration. This is done in various ways, e.g., crushing the spermatic cord, retaining the testes in the inguinal ring, or use of a rubber band, placed around the neck of the scrotum, to cause sloughing off of the scrotum and testes. However, most of these "mechanical" castration methods have proven to be undesirable in one respect or another; for example they (1) are traumatic, (2) may introduce the danger of anesthesia and specialized instruments, (3) are apt to produce infection, and (4) require trained personnel. Moreover, all such mechanical castration methods result in

complete abolition of the testes and this of course implies complete removal of the anabolic effects of any steroids which are produced by the testes and which act as stimuli to growth and protein deposition.

These drawbacks have caused consideration of various alternative sterilization techniques such as the use of chemical sterilization agents. One approach in chemical sterilization involves the use of a cytotoxic agent attached to a molecule that binds to GnRH receptors on gonadotrophs; upon the internalization of the GnRH-cytotoxin conjugate, the cytotoxic agent is released which kills the target cell.

The GnRH-cytotoxin approach is illustrated by the disclosure of WO90/09799, which teaches certain sterilizing agents comprising GnRH analogs coupled to a variety of toxins through an optional linking group consisting of 2-iminothiolane, SPDP (N-succinimidyl-3-(2-pyridyldithio)propionate), bis-diazabenzidine and glutaraldehyde. WO93/15751 also discloses chimeric molecules of GnRH, or analogs thereof, and cytotoxins. The chimera of this disclosure is a molecule in which GnRH peptides are linked via a linker to a modified Pseudomonas exotoxin molecule. At each site of peptide binding to the toxin molecule, there is only one GnRH peptide bound. Administration of such chimeric molecules is reported to result in the destruction of GnRH receptor bearing cells in the pituitary gland, with concomitant reduction in the secretion of sex hormones. The ability of this approach will be determined by the rates of receptor endocytosis and intracellular processing in gonadotrophs. The ultimate result of this process is chemosterilization and reduction of steroid stimulated tumor proliferation. UK Application No. 2,282,812 teaches GnRH attached to a cyclic scaffold containing multiple lysine units, termed a MAP (multiple antigen peptide) or lysine tree, and the scaffold is in turn coupled to a cytotoxin such as Pseudomonas exotoxin. The use of the multi-lysine scaffold permits attaching more than one GnRH per cytotoxin linking site; however, the MAP approach is not necessarily an advantage because MAP conjugates generally have the attribute of high insolubility in

hydrophobic and hydrophilic solvents rendering them more difficult to formulate.

Another approach in animal sterilization involves the use of GnRH vaccines, i.e., immunosterilization. Typically a GnRH molecule, which is only weakly immunogenic, is coupled to an immunogenic macromolecule, such as a protein, in order to enhance the immunogenicity of GnRH; alternatively, a fusion protein containing a GnRH and an immunogenic peptide may be constructed for the same purpose. Animals administered the conjugate or fusion protein develop antibodies against GnRH, which down regulate the action of GnRH resulting in the drastic reduction of sex hormones and the atrophy of hormone dependent organs. A number of GnRH conjugates or fusion proteins suitable for use as vaccines have been described.

A commercial GnRH vaccine is currently being marketed in Australia by Arthur Webster & Co. Pty Ltd under the name of Vaxstrate® for use in cattle (see e.g., R. M. Hoskinson et at, Aust. J. Biotechnol., 1990, 4:166). This vaccine, which is reported to consist of GnRH conjugated to ovalbumin, is poorly immunogenic. This vaccine formulation in mineral oil and DEAE-dextran produces severe injection site reactions and lesions.

US 4,975,420 discloses immunosterilants comprising a GnRH analog in which the amino acid 1, 6 or 10 has been replaced by cysteine, coupled to a carrier protein.

WO88/05308 discloses immunoneutering compositions containing penta-, hexa-, or heptapeptide fragments of native GnRH conjugated with an immunogenic protein.

WO93/08290 describes fusion proteins comprising GnRH and a leukotoxin polypeptide. The leukotoxin serves as a carrier protein to increase the immunogenicity of the antigen.

EP 578,293 discloses fusion proteins comprising a part of an E. coli P-fimbrial filament and GnRH. This carrier system is said to be capable of eliciting a greatly improved immune response against GnRH, and when used in a vaccine, avoids the need for aggressive adjuvants such as complete/incomplete Freunds adjuvant (CFA/IFA).

WO92/19746 teaches recombinant polypeptides comprising GnRH, at least one T-cell epitope and a purification site.

WO90/02187 discloses fusion proteins comprising hepatitis B surface antigen and GnRH. The constructs are said to be sufficiently immunogenic to render unnecessary the use of adjuvants and multiple injections.

US 5,324,512 teaches GnRH linked through an N-terminal glutamine to a carrier protein. The conjugates are claimed to be useful as antifertility vaccines and in the treatment of prostate cancer.

WO94/25060 discloses immunogenic peptide containing GnRH, a T-cell epitope and, optionally, an invasin domain. The peptides are useful as antifertility vaccine and for treating androgen-dependent carcinoma.

UK 2,228,262 discloses conjugates in which [D-Lys$^6$]GnRH (i.e. amino acid 6 (glycine) of native GnRH has been replaced by D-Lys) is linked to a carrier protein through the ε-amino group of the D-Lys. The conjugates may be used to control fertility or for therapy of prostate cancer.

As previously discussed, Pseudomonas exotoxin has been coupled to GnRH, and the resulting construct used for the destruction of gonadotrophs; the GnRH of the construct acts to deliver the toxin into cells bearing GnRH receptors, and once inside the cell, the toxin is released and exerts its cytotoxic activity to effect cell killing. The strategy of using a receptor binding ligand to deliver Pseudomonas toxin into the target cells has been well documented with a number of ligands other than GnRH.

Chaudhary, et al., PNAS USA 84:4538-4542 (1987) teach that hybrid fusion proteins formed between PE-40 and transforming growth factor-alpha and produced in bacteria using recombinant DNA techniques will bind to and kill human tumor cells possessing epidermal growth factor receptors.

Edwards, et al., Mol. Cell. Biol. 9: 2860-2867 (1989) describe the preparation of the modified TGF-alpha - PE-40 hybrid

molecules that have been found to have utility in treating bladder tumor cells.

Heimbrook, et al., Proc. Natl. Acad. Sci. USA 87: 4697-4701 (1990) describe the in vivo efficacy of modified TGF-alpha - PE-40 in significantly prolonging the survival of mice containing human tumor cell xenografts.

U.S. patent 4,545,985 teaches that Pseudomonas exotoxin A can be chemically conjugated to an antibody or to epidermal growth factor. While this patent further teaches that these conjugates can be used to kill human tumor cells, these chemically linked toxins have been shown to have undesirable levels of nonspecific activity.

Bailon, Biotechnology, pp. 1326-1329 Nov. (1988) teach that hybrid fusion proteins formed between PE-40, a truncated variant of PE exotoxin A, and interleukin 2 and produced in bacteria using recombinant DNA techniques will bind to and kill human cell lines possessing interleukin 2 receptors.

The use of Pseudomonas exotoxin to increase immunogenicity of a hapten is described in WO92/12173, which teaches fusion proteins of Pseudomonas exotoxin and specific regions of human P-glycoproteins; these fusion proteins are used to raise antibodies against P-glycoprotein. Conjugate vaccines composed of *Staphylococcus aureus* capsular polysaccharide and recombinant protein derived from Pseudomonas exotoxin A is reported in Fattom, A. et al, Inf. Immun., 1993, 61(3):1023-1032.

European patent application 0 261 671 teaches that a portion of the Pseudomonas exotoxin A protein can be produced which lacks the cellular binding function of the whole Pseudomonas exotoxin A protein but possesses the translocating and ADP ribosylating functions of the whole Pseudomonas exotoxin A protein (mw 66,000). The portion of the Pseudomonas exotoxin A protein that retains the translocating and ADP ribosylating functions of the whole Pseudomonas exotoxin A protein is called (mw 40,000) PE-40. PE-40 consists of amino acid residues 253-613 of the whole Pseudomonas exotoxin A protein as defined in Gray, et al., PNAS USA 81:2645-2649 1984. This patent application further

teaches that PE-40 can be linked to transforming growth factor-alpha to form a hybrid fusion protein produced in bacteria using recombinant DNA techniques.

The conjugates of the present invention differ from those reported in that they employ a hydrophilic linear scaffold as a means to increase loading of desirable haptens onto a carrier protein. The commercialization of synthetic conjugate vaccine products has changed in recent years to require more and more vigorous charactarizations of vaccine components, called here for convenience the "vaccine family". A vaccine family is a group of closely related structures comprising the carrier protein covalently coupled to incremental numbers of peptide haptens, i.e Z-Xn + Z-Xn+1 + Z-Xn+2... to a limit of Z-Xn+4 where n+r is the nubmer of chemically available linking sites on the carrier protein. The conjugates of the instant invention have an advantage in that the scaffolds bearing multiple GnRH haptens have higher molecular weight enabling easier analytical charactarization of the vaccine family. Simple GnRH carrier protein conjugate vaccine families cannot be easily resolved by conventionally available analytical methods such as gel electrophoresis, gel exclusion or ion-exchange chromatography. Thus compared to conventional GnRH conjugates they provide more GnRH per carrier molecule while utilizing a minimal number of carrier molecule attachment sites, thereby reducing the size of the family of conjugate molecules produced; and compared to GnRH-MAP conjugates such as those described in UK 2,282,812 they are more water soluble and therefore more suited for vaccine or pharmaceutical formulation. The present conjugates have been found to possess unexpected potency in stimulating an anti-GnRH immune response.

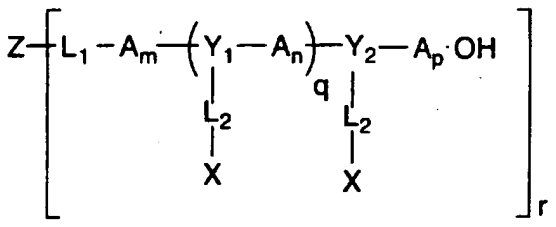
## SUMMARY OF THE INVENTION

The present invention relates to conjugates which comprise at least two GnRH linked to a linear oligopeptidyl scaffold, which is in turn coupled to an immunogenic carrier protein. The conjugates of the present invention are useful as vaccines for use in immunosterilizing animals, fertility control, and for treatment of steroid hormone stimulated

tumors or conditions such as endometriosis. The present invention further concerns a vaccine formulation comprising a GnRH-conjugate in an oil-in-water emulsion, and optionally containing an immune response enhancer.

DETAILED DESCRIPTION OF THE INVENTION

The conjugates of the present invention may be represented by the general formula I:



I

wherein

A is independently an amino acid selected from Gly, Ser, Thr, β-Ala and Ala, with the proviso that at least one A is Ser or Thr;

$L_1$ is a linker optionally attached to an internal marker;

$L_2$ is independently a linker;

X is a GnRH derivative modified at positions 1, 6 or 10 for linker enablement;

$Y_1$ and $Y_2$ are independently Lys or Orn;

Z is an immunogenic carrier protein;

m is 0 to 3;

n is 1 to 10;

p is 0 to 1;

q is 1 or 2;

r is 1 to 10.

Preferred embodiments are where:

(1) the immunogenic carrier protein, Z, is selected from Pseudomonas exotoxin, or a variant thereof, and ovalbumin; or

(2) the GnRH moiety, X, is represented by the sequence [SEQUENCE ID NO.: 1]:

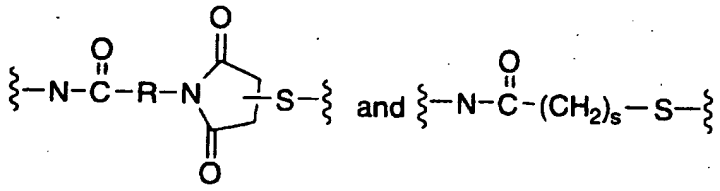
(B)pQ-His-Trp-Ser-Tyr-W-T-Arg-U-V
1 2 3 4 5 6 7 8 9 10

wherein:

| | | |
|---|---|---|
| B is | a thiol containing linker of the formula $HS-(CH_2)_n-CO-$; |
| p is | 0 or 1; |
| n is | 1 to 10; |
| Q is | pGlu or Gln; |
| W is | a D- or L- amino acid selected from glycine, alanine, cysteine, homocysteine, ornithine or lysine; |
| T is | Leu or Nle; |
| U is | Pro or 4-hydroxy-Pro; and |
| V is | Gly-$NH_2$, D-Ala-$NH_2$, NH-Et, NH-Pr or Arg-Gly-$NH_2$; |

with the proviso the GnRH is linked to $L_2$ via an amino or a sulfhydryl group on Q or W; or

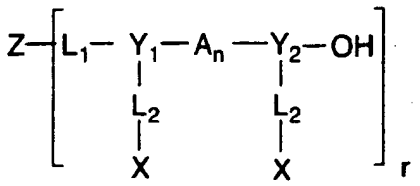
(3) $L_1$ and $L_2$ are independently selected from



wherein:

| | | |
|---|---|---|
| R is | $C_1$-$C_5$ alkyl, phenyl or $C_5$-$C_6$ cycloalkylene; |
| s is | 1 or 2; |
| $L_1$ is | attached to βAla; and |
| Z is | selected from Pseudomonas exotoxin, or a variant thereof, and ovalbumin; or |

(4) the conjugate is represented by the formula



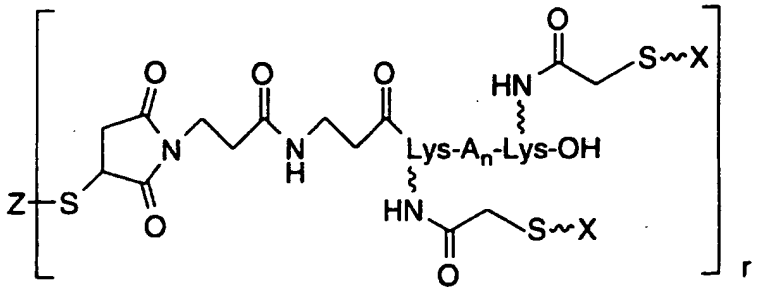
wherein

n is 3 to 8;

r is 1 to 3; and

A, X, $Y_1$, $Y_2$, Z, $L_1$ and $L_2$ are as defined under formula I.

An even more preferred conjugate has the formula:



wherein

one of A is Ser or Thr, and the others are selected from Gly, Ala and β-Ala;

X is a GnRH having a free sulfhydryl group;

Z is as defined above;

n is 5 or 6; and

r is 1 to 3.

Preferably $A_n$ is [SEQUENCE ID NO:10] Gly-Gly-Ser-Gly-Gly or [SEQUENCE ID NO:11] Gly-Gly-Thr-Gly-βAla-Gly.

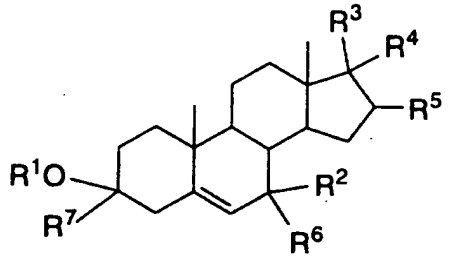
In another aspect, the present invention provides a vaccine composition comprising a GnRH-conjugate in an oil-in-water emulsion vehicle which comprises:

(a) a metabolizable oil;

(b) a non-ionic surfactant; and

(c) an emulsifier.

Preferably, the vaccine composition futher comprises an immunopotentiating amount of an immune response enhancer having the formula 1:




1

wherein

$R^1$ is H, $C_{2-8}$ alkenyl, $C_{1-8}$ alkyl, benzyl, phenyl or $COR^4$, wherein $R^4$ is H, $C_{1-8}$ alkyl, $C_{2-8}$ alkenyl, benzyl or phenyl wherein the phenyl moiety may have up to three substituents selected from the group consisting of hydroxy, carboxy of 1-4 carbon atoms, halo, $C_{1-4}$ alkoxy, $C_{1-4}$ alky, and $C_{2-4}$alkenyl, $SO_3M$ or $PO_3M$, wherein M is H or sodium or potassium;

$R^2$ is H or $OR^1$;

$R^3$ is $OR^1$ or $R^3$ and R4 together form an oxo;

$R^4$, $R^5$, $R^6$, and $R^7$ are independently H or methyl;

with the proviso that when $R^3$ and R4 together form an oxo, $R^5$, $R^6$, $R^7$ and $R^2$ are each H; and that when $R^2$ is H, $R^4$, $R^5$, $R^6$ and $R^7$ are each hydrogen, and $R^3$ is $OR^1$.

The meaning of each A in the above formulae is independent of that of the others; thus, for example for $A_m$ in which m is 3, each of the three amino acid residues may be the same as or different from the others, or the A in $A_n$ and $A_m$ may be the same or different.

Standard three-letter symbols (except in some formulae in the Examples where one letter symbols are used for compactness) are used to denote the standard amino acids. Unless otherwise specified, an amino acid encompasses both the L- and D- forms. In the various

formulae used throughout the application, OH indicates the carboxyl terminus of a peptide.

The following abbreviations are used:

| | |
|---|---|
| CZE | capillary zonal electrophoresis |
| DCC | dicyclohexylcarbodiimide |
| DIEA | diisopropylethylamine |
| DMF | dimethylformamide |
| DTT | dithiothreitol |
| EDTA | ethylenediamine tetraacetic acid, disodium salt |
| FMOC | 9-fluorenylmethoxycarbonyl |
| HOBt | 1-hydroxybenzotriazole |
| HPSEC | high performance size exclusion chromatography |
| MPS | 3-maleimidopropionic acid N-hydroxysuccinimide ester |
| PE | Pseudomonas exotoxin |
| PyBOP | benzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate |
| SDS-PAGE | sodium dodecylsulfate polyacrylamide gel electrophoresis |
| TFA | trifluoroacetic acid |

Unless otherwise specified, the following definitions are used in this application.

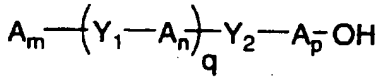
"Alkyl", "alkenyl" and "alkynyl" means a linear or branched hydrocarbon chain having the indicated number of carbon atoms that is saturated, has one or more double bonds, and has one or more triple bonds, respectively.

"Halo" means fluorine, chlorine, bromine or iodine.

The term "GnRH", as used herein throughout the application, is intended to encompass the native GnRH and analogs or derivatives thereof that are capable of eliciting anti-GnRH antibodies when administered to a host in accordance with the present invention. When a

particular GnRH molecule is meant, its amino acid sequence will be specified.

The term "scaffold" means the portion of conjugate shown below:



wherein A $Y_1$, $Y_2$, m, n, p and q are as defined above under formula I.

The term "immunogenic carrier protein" means a polypeptide or a protein to which a weak immunogen has been covalently bound that stimulates a strong immune response to the normally weak immunogen.

The term "internal marker" means an unnatural amino acid that has been incorporated to facilitate characterization (by amine acid analysis) of the conjugate and the calculation of the ratio of GnRH to carrier protein.

"GnRH-conjugate" means generically a GnRH coupled to an immunogenic carrier protein, with or without the scaffold described herein.

The term "immunopotentiating amount" means an amount effective to increase the antibody titre over that normally raised against an immunogen in the absence of an immune response enhancer.

The conjugates of formula I of the present invention are essentially made up of three components: a GnRH, a lysine-containing oligopeptidyl scaffold to which two or more GnRH molecules are attached, and an immunogenic carrier protein to which one or more scaffolds are linked. Each of the components will be discussed in detail hereinbelow.

Native GnRH, also known as luteinizing hormone releasing hormone (LHRH), is a decapeptide having the amino acid sequence [SEQUENCE ID NO.: 2]:

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-$NH_2$

in which pGlu is pyroglutamate. Many analogs or derivatives of native GnRH have been reported, and they may be obtained by addition, deletion, replacement or other alterations to the constituent amino acids of the native GnRH. Non-limiting examples of GnRH analogs or derivatives that may be suitable for use in the present inventions include those disclosed in UK Patent 2,228,262 (e.g. [D-Lys6]GnRH); US Patent 4,975,420 (e.g. [D-Cys$^6$]GnRH); US Patent 4,608,251 (e.g. N-terminus modified nonapeptide or decapeptide); European Patent Application 464,124 (e.g two GnRH in tandem); European Patent Application 293,530 (e.g. C-terminus extended GnRH); PCT Application 88/05308 (e.g. truncated fragments of GnRH); and US Patent 5,324,512 (pGlu of native GnRH replaced by Gln).

Preferably, the native GnRH is modified to include an amino acid that provides a functional group through which the GnRH can be linked to the scaffold core; such an amino acid may be located at the N- or C-terminus, or it may replace amino acid 6 (Gly) of the native GnRH. More preferably, the GnRH includes a free amino or sulfhydryl group. Free amino group may be obtained by, for example, replacing the N-terminal pGlu with Gln, or by replacing Gly$^6$ of the native GnRH with Lys. Free sulfhydryl group may be obtained by replacing one of the amino acids, for example, the amino acid at position 1, 6 or 10, with cysteine; or alternatively, a free amino group may be thiolated using homocysteine thiolactone or mercaptopropanoic acid to provide the free sulfhydryl group.

In a preferred embodiment, GnRH may be represented by the sequence [SEQUENCE ID NO.: 1]:

$$(B)_p\text{-Q-His-Trp-Ser-Tyr-W-T-Arg-U-V}$$

wherein:

| | |
|---|---|
| B is | a thiol containing linker of the formula $HS\text{-}(CH_2)_n\text{-}CO\text{-}$; |
| p is | 0 or 1; |

| | |
|---|---|
| n is | 1 to 10; |
| Q is | pGlu or Gln; |
| W is | a D- or L- amino acid selected from glycine, alanine, cysteine, homocysteine, ornithine, and lysine; |
| T is | Leu or Nle; |
| U is | Pro or 4-hydroxy-Pro; and |
| V is | Gly-$NH_2$, D-Ala-$NH_2$, NH-Et, NH-Pr or Arg-Gly-$NH_2$; |

with the proviso that at least one of Q or W has a free amino or sulfhydryl group though which GnRH is linked to $L_2$.

More preferred GnRH are of the formula [SEQUENCE ID NO.: 3]:

(B)p-Q-His-Trp-Ser-Tyr-W-Leu-Arg-Pro-Gly-$NH_2$
1 2 3 4 5 6 7 8 9 10

wherein B, p, Q and W are as defined above.

The scaffold is a linear oligopeptide having up to a total of twenty seven amino acids in the sequence with at least two of those being independently ornithine or lysine. The other amino acids are selected from amino acids of small size with at least one of the non-Lys non-Orn amino acids being a hydrophilic amino acid. Examples of suitable small amino acids are alanine, β-alanine, glycine, serine, and threonine; serine and threonine are in addition hydrophilic. The hydrophilic amino acid is preferably serine or threonine, and up to 5 hydrophilic amino acid residues may be incorporated into the scaffold. More preferably, the scaffold oligopeptide has from 5 to 10 amino acids, one or two of which are Ser or Thr. The lysine/ornithine residues are separated from each other by at least one amino acid, preferably the spacer between lysine/ornithine residues is about 3 to 8 amino acids.

In a preferred embodiment, the scaffold core has the sequence:

ß-Ala-Lys-$A_n$-Lys

wherein n is 4 to 7, more preferably 5 or 6; one of A is Ser or Thr, and the others are selected from Gly, Ala and βAla. In a more preferred embodiment, the scaffold core has the amino acid sequence [SEQUENCE ID NO: 4]:

ß-Ala-Lys-Gly-Gly-Ser-Gly-Gly-Lys.

In another more preferred embodiment, the scaffold has the amino acid sequence [SEQUENCE ID NO.:5]:

ß-Ala-Lys-Gly-Gly-Thr-Gly-βAla-Gly-Lys.

"Immunogenic carrier protein" may be any one that helps to elicit a strong immune response to a normally weak immunogen. Examples of carrier protein that have been disclosed as being potentially useful to enhance immunogenicity of GnRH include keyhole limpet hemocyanin, tetanus toxoid, diphtheria toxoid, ovalbumin, human or bovine serum albumin, purified protein derivative of tuberculin, thyroglobulin, outer membrane protein complex of Neisseria meningitidis (OMPC); and the like. In addition, the present inventors have discovered that Pseudomonas exotoxin, or a variant thereof, and urease may also be suitably employed as the immunogenic carrier protein in the present invention. Urease has not been heretofore known to be suitable as immunogenic carrier protein, and Pseudomonas exotoxin has only been used in conjunction with GnRH as a cytotoxic agent, i.e., for cell killing, and not as immunogenicity enhancer. In a preferred embodiment of the present invention, the carrier protein is selected from Pseudomonas exotoxin or a variant thereof, and ovalbumin.

Pseudomonas exotoxin is a protein composed of 613 amino acids arranged into 3 major, and one minor domain. The preferred Pseudomonas exotoxins are variants thereof having decreased toxicity, for example, segments of Pseudomonas exotoxin wherein the binding or the ADP ribosylating activity has been attenuated or inactivated through deletion or partial deletion, insertion or substitution of amino acids in the

binding or ribosylating domain, or where the PE holotoxin has been inactivated, for example by photoinactivation. The efficacy of PE conjugates as immunogen or vaccine is independent of the toxin activity of the PE; thus a PE-GnRH conjugate may be inactivated, e.g. by photoinactivation, and still retain its immuogenic properties . One example of a Pseudomonas exotoxin with decreased toxin activity has had amino acids 1-252 deleted, which comprise most or all of the binding region and retaining amino acids 253-613 which contain the cell translocation region and the toxin region. This Pseudomonas exotoxin fragment has been identified as PE-40 - See Hwang et al., infra, Kondo et al J. Biol Chem 263 pg 9470-9475 (1988), Chaudhary et al, PNAS-USA, 87 pg 308-312 (1990) and US Patent 4892827 to Pastan et al.

The Pseudomonas exotoxin fragment PE-40 has been further modified by removing additional amino acids 365-380 to provide PE-38. PE-40 and PE-38 may be further modified by adding lysine containing oliogopeptide fragments to their N-termini. Addition of the 10 amino acid peptide Met-Ala-Asn-Leu-Ala-Glu-Glu-Ala-Phe-Lys (the "Lys" peptide) to the N-terminus of PE-40 and PE-38 produces Pseudomonas exotoxins identified as Lys PE-40 and Lys PE-38, respectively; addition of the 11 amino acid peptide Met-Leu-Gln-Gly-Thr-Lys-Leu-Met-Ala-Glu-Glu (the "NLys" peptide) produces Pseudomonas exotoxins identified as NLys PE-40 and NLys PE-38, respectively.

Replacing PE-38 lysines at 590 and 606 with glutamine, and lysine 613 with arginine generates the Pseudomonas exotoxin identified as PE-38QQR. Lys PE-38QQR and NLys PE-38QQR have, at their N-termini, the "Lys" and "NLys" peptides, respectively.

The various Pseudomonas exotoxin fragments are prepared using the techniques of biotechnology and recombinant DNA, and are described in Debinski and Pastan, Bioconjug. Chem., 1994, 5(1):40-46, and references cited therein.

The amino acid sequence of NLys PE-38QQR is shown below [SEQ ID No.: 6]; the underlined 4 amino acids represent the N-termial amino acids of PE-38:

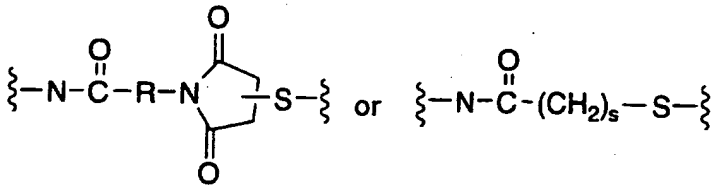
Met Ala Glu Gly....

Met Leu Gln Gly Thr Lys Leu Met Ala Glu Glu Gly Gly
Ser Leu Ala Ala Leu Thr Ala His Gln Ala Cys His Leu
Pro Leu Glu Thr Phe Thr Arg His Arg Gln Pro Arg Gly
Trp Glu Gln Leu Glu Gln Cys Gly Tyr Pro Val Gln Arg
Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser Trp Asn
Gln Val Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro
Gly Ser Gly Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln
Pro Glu Gln Ala Arg Leu Ala Leu Thr Leu Ala Ala Ala
Glu Ser Glu Arg Phe Val Arg Gln Gly Thr Gly Asn Asp
Glu Ala Gly Ala Ala Asn Gly Pro Ala Asp Ser Gly Asp
Ala Leu Leu Glu Arg Asn Tyr Pro Thr Gly Ala Glu Phe
Leu Gly Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly
Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln Ala His
Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val Gly Tyr
His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe
Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile
Trp Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala
Tyr Gly Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly
Arg Ile Arg Asn Gly Ala Leu Leu Arg Val Tyr Val Pro
Arg Ser Ser Leu Pro Gly Phe Tyr Arg Thr Ser Leu Thr
Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu Arg Leu
Ile Gly His Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr
Gly Pro Glu Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu
Gly Trp Pro Leu Ala Glu Arg Thr Val Val Ile Pro Ser
Ala Ile Pro Thr Asp Pro Arg Asn Val Gly Gly Asp Leu
Asp Pro Ser Ser Ile Pro Asp Gln Glu Gln Ala Ile Ser
Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly Gln Pro Pro
Arg Glu Asp Leu Arg

Other suitable Pseudomonas exotoxins include photoinactivated holotoxin and PE-38 (incluiding Lys PE38, NLys PE-38, PE-38 QQR, Lys PE38QQR, and NLys PE-38QQR) in which the disulfide bond has been reduced.

The more preferred immunogenic carrier protein for the present invention is NLys PE-38QQR or ovalbumin.

The coupling of the scaffold to the carrier protein, and the GnRH to the scaffold is achieved through linkers $L_1$ and $L_2$, respectively. The "linker" may be derived from any heterobifunctional cross-linking agents carrying functionalities that are reactive with amino group and sulfhydryl group; for examples, SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate), glutaraldehyde, iminothiolane, bromoalkanoic anhydrides, maleimido-benzoyl-N-succinimide ester, 3-maleimidopropionic acid N-hydroxysuccinimide ester, and the like may be used. Preferred linkers $L_1$ and $L_2$ have the formula:



wherein:

R is $C_1$-$C_5$ alkylene, phenyl or $C_5$-$C_6$ cycloalkylene; and

s is 1 or 2.

The N and S at the termini originate from the peptides to be linked. In a preferred embodiment R is -$CH_2CH_2$- and s is 1.

The linker $L_1$ preferably includes an internal marker. "Internal marker" is an unnatural amino acid incorporated to facilitate characterization of the conjugate and the calculation of the ratio of GnRH to carrier protein. Without a marker it would be difficult to analyze for amino acid content because there is no way of knowing how many of the GnRH peptides bond with the carrier protein since the carrier protein generally has more than one site with which the GnRH can bond. Incorporation of a marker also aids in the purification of the conjugate from small molecules, which are deleterious in that they can set up a suppressor response in the vaccinated individual to reduce the efficacy of

the vaccine. Examples of suitable markers include β–Ala and Nle; preferably β-Ala is used.

Conjugates of this invention are constructed by:

a) synthesizing the oligopeptidyl scaffold and purifying this component;

b) synthesizing and then purifying the GnRH component;

c) coupling the purified scaffold component of step (a) with the purified GnRH of step (b) via a linker, and purifying the GnRH loaded scaffold thus formed; and

d) coupling the GnRH loaded scaffold of step (c) with an immunogenic carrier protein via a linker to form the conjugate of the present invention.

The order of the foregoing steps need not be precisely as described above. For example, the scaffold may be linked to the carrier protein before coupling the GnRH components. The heterobifunctional linker may be attached first to either one of the two components to be coupled. Thus, the conjugate may be constructed linearly by reacting the carrier protein with a linking group to form Z-$L_1$', which is reacted with the scaffold to form Z-$L_1$-scaffold, which is reacted with a second linking group to form Z-$L_1$-scaffold-$L_2$', which is then reacted with the GnRH component to form the conjugate of formula I. This linear construction can of course start at the other end, i.e., starting from GnRH, and attaching the carrier protein as the last step. The conjugate of formula I may also be constructed by joining Z with $L_1$'-scaffold, followed by $L_2$'-GnRH, or by joining Z-$L_1$' with scaffold, followed by $L_2$'-GnRH. $L_1$' and $L_2$' signify that the linking group still has one unreacted functional group. Other variations of constructing the conjugates of the present invention will be apparent to those of ordinary skill in the art.

Alternatively, the whole GnRH-scaffold construct could be prepared on resin. Synthesis of branched peptides on resin is known. Therefore, synthesis of GnRH-scaffold may be accomplished using methods generally known to one of ordinary skill in the art.

The oligopeptides, i.e., the scaffold and the GnRH components, may be prepared by standard solid phase peptide synthetic methods using a peptide synthesizer. We have discovered that it is desirable to purify the oligopeptides before coupling reaction. The

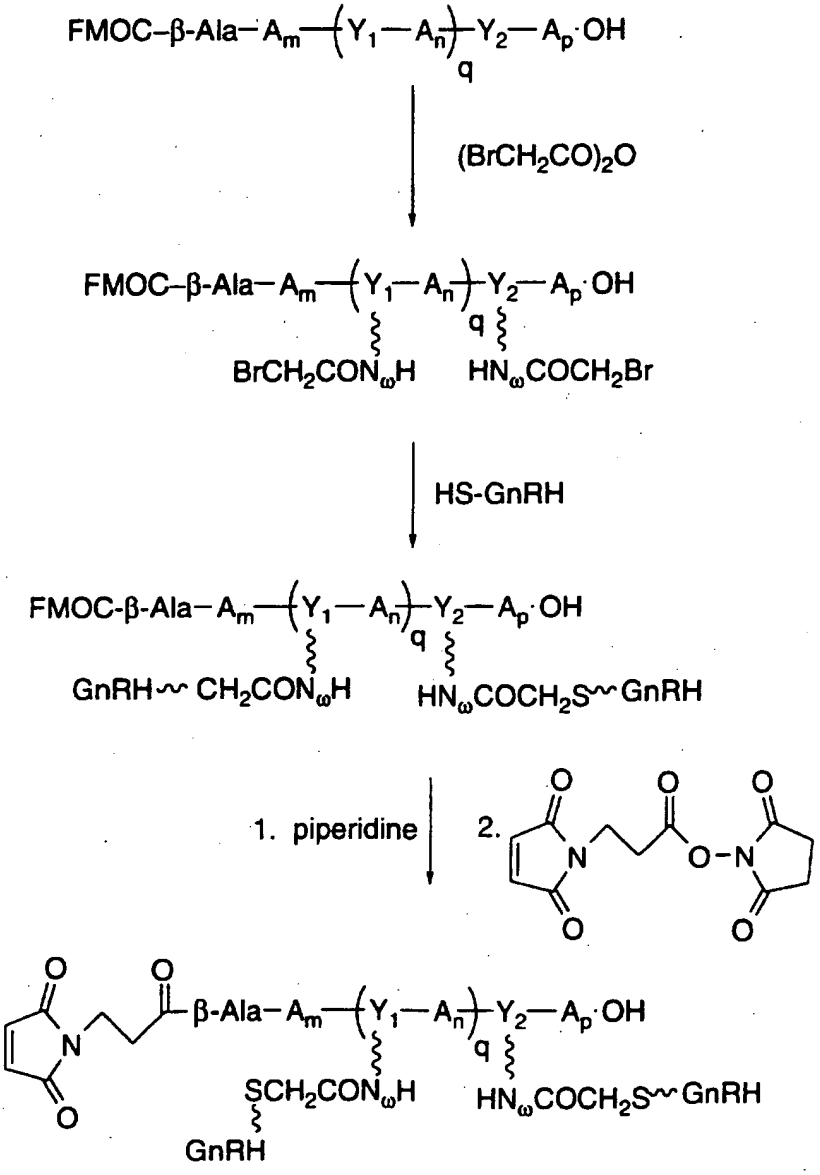
oligopeptides may be purified according to standard peptide purification procedures known in the art. A particularly preferred method is to use chromatographic methods including reverse-phase high performance liquid chromatography (RPHPLC), or by affinity chromatography using GnRH affinity media such as an anti-GnRH antibody column.

The carrier proteins are either commercially available, or they can be prepared according to methods known in the art. The various Pseudomonas exotoxin fragments are prepared using techniques of biotechnology and recombinant DNA; these fragments and their methods of preparation are described in Debinski and Pastan, Bioconjug. Chem., 1994, 5(1):40-46, and references cited therein.

Peptide-peptide (including peptide-protein) linkage may be accomplished in a variety of different ways. Thus, the GnRH may be linked to the scaffold core, and the scaffold core linked to the carrier protein, using known conventional cross-linking agents as previously described. As one example of suitable linking methods, the free amino groups of a first peptide are bromoacylated, or maleimidated. The thus activated first peptide may then be reacted with a second peptide bearing free sulfhydryl group; the free sulfhydryl group may be provided by thiolating free amine on the peptide with a reagent such as N-acetylhomocysteine thiolactone, or by a cysteine, whether native to or deliberately introduced into the peptide, or from the reduction of disulfide bonds in the peptide. Other methods conventionally used in peptide linker chemistry are also suitably used in the present invention; essentially any method where nucleophilic and electrophilic groups are provided on the reacting partners is sufficient to achieve linkage of peptides.

The reaction schemes below illustrate schematically one method outlined above for making the conjugates of the present invention. In the schemes, the variables are as defined under Formula I.

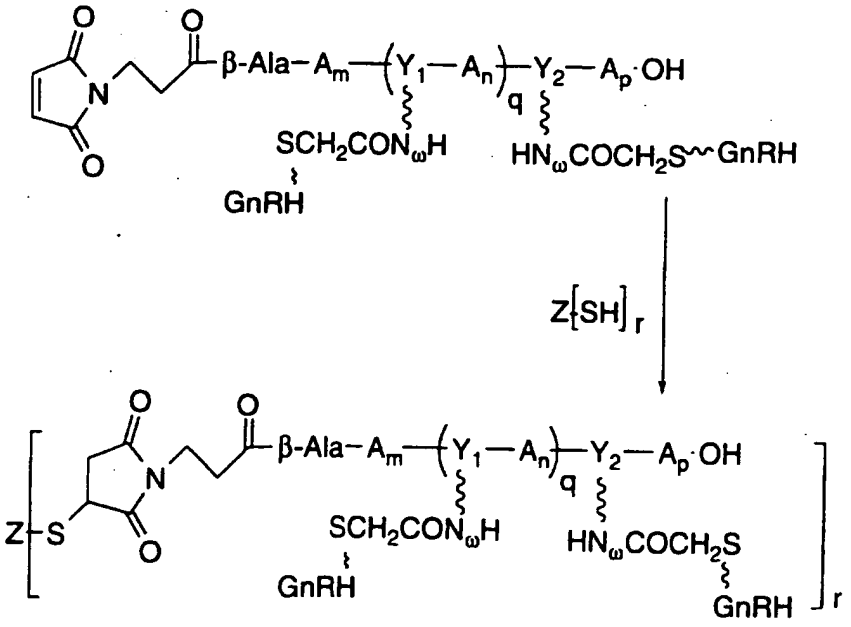
SCHEME 1




In Scheme 1, the scaffold bearing a protected internal marker β-alanine is reacted with bromoacetic anhydride to effect bromoacetylation of the terminal amino groups ($N_\omega$) of the $Y_1$ and $Y_2$ residues of the scaffold. The reaction is carried out under nitrogen in an

inert organic solvent such as methylene chloride, dimethylformamide or a combination thereof, typically at ambient temperature. The bromoacetylated scaffold is reacted with a GnRH having a free sulfhydryl group, for example [DCys$^6$]GnRH or HSCH$_2$CH$_2$CO-[Gln$^1$]GnRH, resulting in scaffold supported GnRH. The reaction is carried out in aqueous acetonitrile at room temperature, and the pH of the reaction mixture is preferably maintained at about 8, and generally between about 7.5 and about 8.5. The Fmoc protecting group is removed using e.g., piperidine, and the deprotected peptide is reacted with a maleimidyl alkanoic acid activated ester such as MPS, at room temperature in an inert organic solvent such as dimethylformamide, and in the presence of a base such as diisopropylethylamine, to provide maleimidated scaffold supported GnRH.

SCHEME 2




In Scheme 2, the carrier protein having a free thiol is reacted with the scaffold-supported GnRH product shown in Scheme 1 to provide the desired conjugate. The free thiol may be associated with a free

cysteine, or generated by reducing disulfide bond(s) in the carrier protein using, for example, dithiothreitol, or introduced onto the carrier protein by reacting the carrier protein with a thiolating agent, such as N-alkanoylhomocysteine thiolactone. More than one free thiol may be present on the carrier protein, and therefore more than one scaffold-supported GnRH may be loaded onto the carrier protein.

It will be understood that the reaction sequence in the above schemes only illustrates the invention, and can be adapted or modified by one skilled in the art without undue experimentation to arrive at other variations within the scope of the invention.

Conjugates of the present invention have utility in human medicine as well as in veterinary medicine. This follows from the fact that there are several important biological reasons for employing castration and antifertility drugs in humans; for example, the growth of certain breast and prostate cancers is influenced by reproductive hormones and can be restricted by steroid hormone manipulation. Another area of application in human medicine is treatment of endometriosis. This condition, which produces painful growth of endometrial tissue in the female peritoneum and pelvis, also responds to inhibition of sex steroid synthesis. Those skilled in this art will also appreciate that the herein disclosed compounds could be used to partially reduce sex-steroid secretions, and thus reduce or eliminate certain hormone related disease states. Conjugates of the present invention may also be used to control fertility in humans, for example, as a chemical sterilant.

The conjugates of the present invention can also be used in veterinary medicine or animal husbandry for conditions in which it is desirable to reduce or eliminate reproduction and/or reproductive hormone driven behavior, physiology or anatomy. Sterilization of animals has primarily been achieved by surgical removal of the gonads. Surgery necessarily involves some degree of pain, trauma and stress for the animal with the potential for infection and death. In food animals, neutering has been used as a means of controlling undesirable behaviors or meat characteristics but it has resulted in substantial production losses.

In the case of swine, intact males have a much higher feed efficiency (approximately 18%) than castrates. However, androstenone is deposited in the fat of the intact male giving the meat an undesirable smell and odor.

Accordingly, another aspect of the present invention provides a method of sterilizing animals comprising administering to said animal a conjugate of formula I in an amount effective to elicit anti-GnRH antibodies. Vaccination using the conjugates of the present invention eliminates the need for surgical neutering. Therefore, it eliminates the pain, stress, trauma, infection, death, production loss and animal welfare issues associated with surgical neutering. Desirable sequellae of vaccination include transient sterilization, controlling undesirable gonadal steroid hormone driven behavior such as aggression in males and estrus behavior in females, improving feed efficiency and carcass quality in food animals such as swine and cattle and a method for eliminating boar taint in the carcasses of male pigs.

The dose/time adjustments associated with the use of these compounds can vary considerably and will depend on a variety of factors such as the species of animal to be treated, the particular GnRH and/or carrier used, the adjuvant, the age of the animal, and the desired outcome of vaccination. In general, the conjugates are administered by subcutaneous or intramuscular injection into a mammal at a rate of 1 μg to 1000 μg of conjugate per dose . A single dose of the conjugates of the present invention may be all that is required to achieve sterilization, but multiple dises spaced at one to six week intervals are alternative sterilization schemes. Furthermore, as sterilization agents, the compounds of this invention can be used before or after puberty; thus they can delay sterilization, which is especially useful in those areas of animal husbandry where the anabolic benefits associated with the flexibility of timing of non-surgical sterilization can contribute positively to feed efficiency, meat production and/or quality.

In swine, the conjugates can be used to maximize the boar-like growth efficiency and carcass quality while eliminating the offensive odor and taste of boar meat. This can be accomplished with one or two

intramuscular or subcutaneous injections administered at various times during the grow out period. An example of convenient and efficacious schedule consists of an initial vaccination at the time of housing in the grower/finisher facility (9-16 weeks of age) with a booster late in the grow out (between 18 and 22 weeks of age). Each vaccination may be at a dose of about 1 μg to about 1000 μg of the conjugate, preferably about 10 μg to about 100 μg is used. For a single dose regimen, the amount of the conjugate is generally at a higher level than that used for two or more doses.

Feedlot cattle could be treated in a manner similar to that used for swine, with vaccinations at the time of entry into the feedlot and at another time which would be determined by the effect desired, i.e., prevention of pregnancy in the females or growth maximization in the males. Females need to complete the vaccination prior to entering mixed sex housing to prevent pregnancy; however, in housing segregated by sex, vaccination could occur at time of arrival and then 4-12 weeks later to prevent estrus. Bulls should be vaccinated late enough to maximize feed efficiency, but early enough to prevent aggression and to provide marbling of meat.

In companion animals such as dogs and cats, the GnRH vaccine could be administered by subcutaneous or intramuscular injection at times when the standard vaccinations are given (between 6 and 21 weeks of age with a booster at 6 months and annual boosters thereafter).

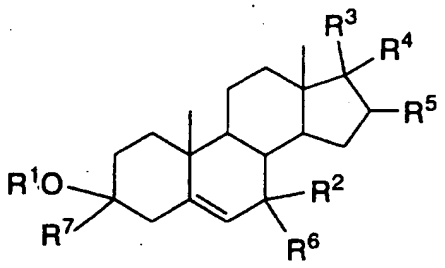
For neutering adult animals such as dogs, cats and horses, two doses administered at 2-8 week intervals followed by and annual boosters should be sufficient to produce neutering. The actual dose and formulation remain to be determined and may vary with the particular conjugate used. However, a dose of 1 to 2000 ug, preferably about 500 ug of an conjugate of the present invention formulated on alum and administered in a volume of 1-3 mls may be sufficiently potent when administered as described above.

In man, conjugates of the present invention can be used to treat sex steroid responsive tumors. Two doses at 1 to 1000 ug per dose of the vaccine can be administered at 2 to 8 week intervals with boosters

at 6 to 12 months until the tumor is eliminated or ceases to be responsive to hormonal therapy.

The conjugates of the present invention can be used for the above-mentioned application without the use of an aggressive adjuvant such as Complete Freund's Adjuvant, which cause injection site lesions and downgrading of feed animal carcasses. Suitable adjuvants are any of those substances recognized by the art as enhancing the immunological response of a mammal to an immunogen without causing an unacceptable adverse reaction, and include aluminum compounds or water in oil emulsions such as Incomplete Freund's Adjuvant (IFA). In a preferred embodiment, the conjugates of the present invention is administered in an oil-in-water emulsion containing a metabolizable oil, a non-ionic surfactant, an emulsifier, and optionally an immune response enhancer of formula 1 as described in detail below.

The present inventors have found that, surprisingly, administration of GnRH-conjugates, with or without the scaffold component disclosed herein, in an oil-in-water emulsion containing a metabolizable oil, a non-ionic surfactant such as a poloxypropylene-polyoxyethylene block polymer, an emulsifier, and optionally an immune response enhancer of formula 1, results in an unexpected high titer of anti-GnRH antibody. Furthermore, animals receiving such a vaccine composition show minimal injection site lesions, as compared to vaccine compositions containing other adjuvants such as IFA. Accordingly, another aspect of the present invention provides a vaccine composition comprising a GnRH-conjugate and an oil-in-water emulsion containging an metabolizable oil, a non-ionic surfactant such as polyoxypropylene (POP)-polyoxyethylene (POE) block polymer, an emulsifier, and optionally an immune response enhancer of formula 1:




1

wherein

$R^1$ is H, $C_{2-8}$ alkenyl, $C_{1-8}$ alkyl, benzyl, phenyl or $COR^4$, wherein $R^4$ is H, $C_{1-8}$ alkyl, $C_{2-8}$ alkenyl, benzyl or phenyl wherein the phenyl moiety may have up to three substituents selected from the group consisting of hydroxy, carboxy of 1-4 carbon atoms, halo, $C_{1-4}$ alkoxy, $C_{1-4}$ alky, and $C_{2-4}$alkenyl, $SO_3M$ or $PO_3M$, wherein M is H or sodium or potassium;

$R^2$ is H or $OR^1$;

$R^3$ is $OR^1$ or $R^3$ and R4 together form an oxo;

$R^4$, $R^5$, $R^6$, and $R^7$ are independently H or methyl;

with the proviso that when $R^3$ and R4 together form an oxo, $R^5$, $R^6$, $R^7$ and $R^2$ are each H; and that when $R^2$ is H, $R^4$, $R^5$, $R^6$ and $R^7$ are each hydrogen, and $R^3$ is $OR^1$.

In the vaccine composition, the metabolizable oil may be an oil of 6 to 30 carbon atoms including alkanes, alkenes, alkynes, and their corresponding acids and alcohols, the ethers and esters therof, and mixtures thereof. The oil may be any vegetable oil, fish oil, animal oil or synthetically prepared oil which can be metabolized in the body of the subject to which the adjuvant is administered, and which is not toxic to the organism. Examples of vegetable oil include peanut oil, soybean oil, coconut oil, olive oil, safflower oil, cottonseed oil, sunflower seed oil, sesame seed oil, and corn oil. Animal oils are usually solids at physiological temperature; however, fatty acids are obtainable from animal fats by partial or complete triglyceride saponifiction which provides the free fatty acids. Most fish contain metabolizable oils which

may be readily recovered. For example, cod liver oil and, shark liver oils, and whale oil such as spermaceti exemplify several of the fish oils. Whale oil such as spermacti may also be used. A number of branched chain oils are synthesized biochemically in 5-carbon isoprene units and are generally referred as terpenoids. Shark liver oil contains a branched, unsaturated terpenoids known as squalene. Squalane, the saturated analog of squalene is a particularly preferred oil for the present invention. The oil component of the adjuvant compositions and vaccines of the invention will usually be present in an amount between 1% and 10%, but preferably in an amount between 2.5 and 5%.

The term "polyoxypropylene-polyoxyethylene block polymer" refers to a polymer made by the sequential addition of propylene oxide and then ethylene oxide to a low molecular weight, reactive compound, usually propylene glycol. These block polymers can be prepared by the methods set out in U.S. Pat. No. 2,674,619 issued to Lunsted, and are commercially available from BASF-Wyandotte under the trademark Pluronic®. The characteristics of these block polymers are determined by the molecular weight of the POP nucleus and of the percentage POE in the product. The POP section imparts hydrophobic characteristics to the block polymer, while the POE section imparts hydrophilic characteristics.

Pluronic® block polymers are designated by a letter prefix followed by a two or a three digit number. The letter prefixes (L, P, or F) refer to the physical form of each polymer, (liquid, paste, or flakeable solid). The first one or two digits is a code for the average molecular weight of the POP base, while the last digit indicates the amount of POE. For example, Pluronic® L101 is a liquid having a polyoxypropylene base of average molecular weight 3,250, with 10% polyoxyethylene present at the ends of the molecule. The preferred block polymers are those which are liquid over a temperature range between about 15°-40° C. In addition, polymer mixtures of liquid and paste, liquid, paste and flakeable solid or liquid and flakeable solid mixtures which are liquid within the specified temperature range may have utility in this invention.

Preferred block polymers are those having a POP base ranging in molecular weight between about 2250 and 4300 and POE in an amount between about 1 and 30%. More preferred are those polymers wherein POP has a molecular weight falling between 3250 and 4000 and the POE component comprises 10-20%. The Pluronic® block polymers L101, L121 and L122 fall within this definition. Most preferred are the block polymers wherein POP has a molecular weight of 4000 and POE in an amount of 10% or POP has a molecular weight of 3250 and POE in an amount of 10% e.g. Pluronic® block polymers L121 and L101 respectively. The block polymer is preferably used in an amount between 0.001 and 10%, most preferably in an amount between 0.001 and 5%.

The term "emulsifier" refers to non-toxic surface active agents capable of stabilizing the emulsion. There are a substantial number of emulsifying and suspending agents generally used in the pharmaceutical sciences. These include naturally derived materials such as gums, vegetable protein, alginates, cellulose derivatives, phospholipids (whether natural or synthetic), and the like. Certain polymers having a hydrophilic substituent on the polymer backbone have emulsifying activity, for example, povidone, polyvinyl alcohol, and glycol ether-based compounds. Compounds derived from long chain fatty acids are a third substantial group of emulsifying and suspending agents usable in this invention. Though any of the foregoing emulsifiers can be used so long as they are non-toxic, glycol ether-based emulsifiers are preferred. Preferred emulsifiers are non-ionic. These include polyethylene glycols (especially PEG 200, 300, 400, 600 and 900), Span®, Arlacel®, Tween®, Myrj®, Brij® (all available from ICI America Inc., Wilmington, Del.), polyoxyethylene, polyol fatty acid esters, polyoxyethylene ether, polyoxypropylene fatty ethers, bee's wax derivatives containing polyoxyethylene, polyoxyethylene lanolin derivatives, polyoxyethylene fatty glycerides, glycerol fatty acid esters or other polyoxyethylene acid alcohol or ether derivatives of long-chain fatty acids of 12-21 carbon atoms. The presently preferred emulsifier is Tween® 80 (otherwise known as polysorbate 80 or polyoxyethylene 20 sorbitan monooleate), although it should be understood that any of the

above-mentioned emulsifiers would be suitable after lack of toxicity is demonstrated. The emulsifier is usually used in an amount of about 0.05 to about 0.5%, preferably about 0.2 to 1%.

The aqueous portion of the adjuvant compositions of the invention is preferably buffered isoosmotic saline. It is preferred to formulate these solutions so that the tonicity is essentially the same as normal physiological fluids in order to prevent post-administration swelling or rapid absorption of the composition due to differential ion concentrations between the composition and physiological fluids. It is also preferred to buffer the saline in order to maintain a pH compatible with normal physiological conditions. Also, in certain instances, it may be necessary to maintain the pH at a particular level in order to insure the stability of certain composition components, such as the glycopeptides. Any physiologically acceptable buffer may be used herein, but it has been found that it is most convenient to use a phosphate buffer. Any other acceptable buffer such as acetate, Tris, bicarbonate, carbonate, and the like can be used as a substitute for a phosphate buffer. It is preferred to use phosphate buffered saline, or saline buffered with a mixture of phosphate and acetate.

The immune response enhancers of formula 1, are either compounds well known in the art (e.g. dehydroepiandrosterone) or they may be prepared according to the disclosures of US Patent 5,277,907 of R. M. Loria, or WO95/10527 of Neurocrine Biosciences. In a preferred embodiment of the vaccine composition, an immunopotentiating amount of the immune response enhancer is included. More preferably, the immune response enhancer is a compound of formula 1 wherein $R^1$, $R^2$, $R^5$, $R^6$, and $R^7$ are each H, and $R^3$ and $R^4$ together form an oxo group, this compound being dehydroepiandrosterone or DHEA.

In the vaccine composition of the present invention the GnRH-conjugate component may be any GnRH-conjugates capable of eliciting anti-GnRH antibodies in the animal given the vaccine. These may include the scaffold supported GnRH-conjugates of formula I disclosed herein, as well as other GnRH-conjugates generally known in the art such as those disclosed in WO93/15751 (GnRH-Pseudomonas

exotoxin), US 4,975,420, PCT Published Application WO88/05308, US Patent 5,324,512, PCT Application WO94/25060 and UK Patent 2,228,262. In a preferred embodiment, the GnRH-conjugate component is a scaffold supported GnRH-ovalbumin conjugate, a scaffold supported GnRH-Pseudomonas exotoxin conjugate, a simple GnRH-Pseudomonas exotoxin conjugate, or a simple GnRH-ovalbumin conjugate; a preferred subset of simple GnRH conjugates is those prepared using the procedure disclosed in WO93/15751.

In a preferred embodiment of the vaccine composition, the oil-in-water emulsion comprises squalane, Tween® 80 and Pluronic® L121. More preferred, the vaccine includes DHEA as the immune response enhancer. Even more preferably, the GnRH conjugate is a GnRH-ovalbumin conjugate, or a GnRH-NLys PE38QQR conjugate, with or without the scaffold structure described above.

The oil-in-water emulsion adjuvant composition is prepared by emulsification using a mixer to form a homogenous emulsion. Typically, the adjuvant composition is microfluidized prior to adding the GnRH-conjugate; the emulsion is cycled through the microfluidizer about 2-20 times. The GnRH-conjugate is then mixed with the adjuvant composition, and the mixture may be again cycled through the microfluidizer. The immune response enhancer, e.g. DHEA, if included in the vaccine composition, may be added to the adjuvant composition prior to microfluidization, or it may be added after the GnRH-conjugate has been mixed with the adjuvant composition. In the latter case, the entire mixture should be microfluidized again, generally 2-10 times through the microfluidizer. The oily particles in the emulsion preferably have diameters of about 0.03 μm and 0.5 μm, more preferably between 0.05 and 0.2 μm.

The following non-limiting examples are provided to further illustrate this invention.

Preparation 1. [DCys$^6$]GnRH: [SEQUENCE ID NO: 7] pGlu-His-Trp-Ser-Tyr-DCys-Leu-Arg-Pro-Gly-$NH_2$

On an ABI431A Peptide Synthesizer (Fmoc chemistry), rink amide resin (521 mg) was coupled sequentially with Gly, Pro, Arg, Leu, D-Cys, Tyr, Ser, Trp, His, Pyroglutamic acid. The Arg and Gly residues were double-coupled and the other residues were single-coupled. The resin was washed with methanol three times and dried under nitrogen (1.069 g resin = 0.539 g weight gain).

The peptide was cleaved for three hours, the resin was filtered off, and the peptide was dried under vacuum. The residue was triturated with diethyl ether and the precipitated peptide was collected by suction filtration and lyophilized (431.7 mg).

A portion of the peptide (195.5 mg) was dissolved in 0.1% TFA, 10% acetonitrile (3 mL), filtered, and purified by RPHPLC (15-45% acetonitrile over 30 minutes using two 25X10 RCM delta pak C18 columns in tandem. A repeat purification of a second portion of peptide was conducted (190.5 mg). Peak fractions were collected, combined and lyophilized, and an aliquot was analyzed by FAB-MS and amino acid analysis. The predicted mass (1229) and amino acid composition were confirmed.

Preparation 2. 3-(Mercaptopropanoyl)-[$Gln^1$]GnRH: [SEQUENCE ID NO: 8] $HSCH_2CH_2CONH$-Gln-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-$NH_2$

The peptide was prepared, up to the $Gln^1$ residue, on an ABI 431A peptide synthesizer (Fmoc chemsitry) using unloaded Rink Amide MBHA resin (NovaBiochem, 0.25 mmol). The initial Gly residue was double coupled onto the resin. All additional amino acids, except for Arg, were single coupled. A standard double coupling protocol was used for $Arg^8$. The N-terminal Fmoc group was removed from the peptide and the resin dried ($N_2$). The resin was transferred to a manual peptide synthesis vessel with fritted bottom ($N_2$ used for agitation). The resin was suspended in DMF (5 mL) and 3-mercaptopropionic acid (1 mmol) was manually coupled to the amino terminus using PyBOP/HOBt activation (1 mmol PyBOP, 0.1 mmol HOBt) and $N_2$ agitation until a

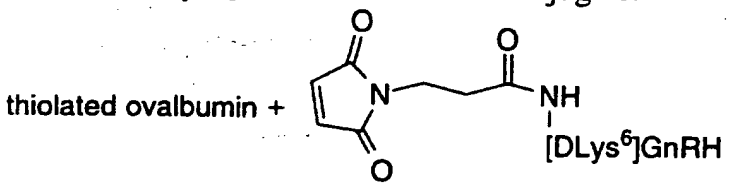
Kaiser test of the resin showed that the reaction was complete (16-48 hr). The peptide was cleaved from the resin with 20 mL of degassed "reagent R" (90:5:3:2 TFA/thioanisole/ethanedithiol/anisole) for 3 hr at RT. The mixture was filtered and the filtrate concentrated *in vacuo*. The remaining residue was triturated with ether and the precipitate collected and dried (272.1 mg). The peptide was purified by RP-HPLC (Delta Pak $C_{18}$, RCM 2-50X10, 45 mL/min.,16->24% $CH_3CN$, 30 min.). The fractions containing the desired product were combined and lyophilized overnight, providing the peptide as a white powder (98 mg, 31%). Electrospray MS showed the expected molecular weight (M+H=1288).

Preparation 3. [(3-Mercaptopropanoyl)-$Gln^1$, $dAla^6$]-GnRH: [SEQUENCE ID NO: 9] $HSCH_2CH_2CONH$-Gln-His-Trp-Ser-Tyr-DAla-Leu-Arg-Pro-Gly-$NH_2$

The peptide was prepared, up to the $Gln^1$ residue, on an ABI 431A peptide synthesizer (Fmoc chemsitry) using unloaded Rink Amide MBHA resin (NovaBiochem, 0.25 mmol). The initial Gly residue was double coupled onto the resin. All additional amino acids, except for Arg, were single coupled. A standard double coupling protocol was used for $Arg^8$. The N-terminal Fmoc group was removed from the peptide (regular piperidine cycle on ABI) and the resin dried ($N_2$). The resin was transferred to a manual peptide synthesis vessel with fritted bottom ($N_2$ used for agitation). The resin was suspended in DMF (5 mL) and 3-mercaptopropionic acid (1 mmol) was manually coupled to the amino terminus using PyBOP/HOBt activation (1 mmol PyBOP, 0.1 mmol HOBt) and $N_2$ agitation until a Kaiser test of the resin showed that the reaction was complete (16-48 hr). The peptide was cleaved from the resin with 20 mL of degassed "reagent R" (90:5:3:2 TFA/thioanisole/ethanedithiol/anisole) for 3 hr at RT. The mixture was filtered and the filtrate concentrated *in vacuo*. The remaining residue was triturated with ether and the precipitate collected and dried (232.0 mg). The peptide was purified by RP-HPLC (Delta Pak $C_{18}$, RCM 2-50X10, 45 mL/min.,15->30% $CH_3CN$, 30 min.). The fractions containing the

desired product were combined and lyophilized overnight, providing the peptide as a white powder (112 mg, 35%). Electrospray MS showed the expected molecular weight (M+H=1303).

Preparation 4. [DLys6]GnRH-ovalbumin Conjugate:




1) Thiolation of Ovalbumin.

A thiolation mixture consisting of 86 mg ethylenediamine tetraacetic acid disodium salt (EDTA) and 15.4 mg of dithiothreitol (DTT) in 10 mL of pH 11 0.1M borate buffer was prepared. Ovalbumin (50.6 mg) was dissolved in this solution and then 49.6 mg of N-acetylhomocysteine thiolactone was added and this solution was filtered through a 0.22 μ filter. The filtrate (9.2 mL) was degassed and after the air replaced by nitrogen was aged for 22 hr in a nitrogen box. This was then dialyzed against three 4L volumes of 0.01M pH 7.06 $PO_4$ buffer with constant nitrogen sparging for 9, 15 and 7 hr respectively affording a solution containing 681 nanomoles of thiol/ mL (Ellman assay). The solution was aged for 24 hr and then used in the next step.

2) Conjugation of thiolated ovalbumin.

The pH of 4.0 mL of the thiolated ovalbumin solution (0.68 μmol SH / mL; 2.72 μmol SH total ) was adjusted to 7.76 with a saturated solution of diisopropylethylamine (DIEA) containing an equal amount of diisopropylethylamine hydrochloride. A coconjugation solution was prepared as follows: 375 mL ( 375 μg; 2.04 μmol) of an aqueous γ-maleimidobutyric acid solution ( 1.0 μg / μL) was mixed with 125 μL (1.25 mg; 0.68 μmol) of an aqueous solution of 6-($N_\varepsilon$-maleimidopropionyl)-d-lys-GnRH (10 μg / mL). There is a total of 2.72 μmoles of maleimide functionality in the coconjugation solution. This

was mixed with vigorous stirring with the above thiolated ovalbumin solution (total of 2.72 μmoles of SH) and aged for 23.5 hr.

3) Purification and analysis.

The reaction mixture was then dialyzed in Spectrapor 2 tubing vs. 4 L of a 0.145 M NaCl solution containing 1 mL of acetic acid (pH 3.64) for 3 hr followed by dialysis in the same solution for 64 hr. . The solution was then analyzed by HPSEC on a TSK 2000 column and found to be free of unconjugated 6-($N_\varepsilon$-maleimidopropionyl)-d-lys-GnRH. Amino acid analysis for β-alanine ( a marker for the GnRH arising from hydrolysis of the maleimidopropionyl moiety) and the constituent amino acids indicates that the conjugate solution contains 0.18 mg of 6-($N_\varepsilon$-maleimidopropionyl)-d-lys-GnRH / ml and 2.57 mg ovalbumin / mL.

Preparation 5. NLys PE38QQR

Plasmid PJH4 (Ref. Hwang. J. "Cell" (1987, 48; 129-136) contains the coding sequence for $PE_{1-613}$. Oligonucleotide directed mutagenesis as described in 15.51-15.73, Molecular Cloning, 2nd ed (1989) edited by Sambrook, Fritch & Maniatis (Cold Spring Harbor Press) has been used as a covenient way to make deletions/mutations in the PE molecule. An NdeI/Hind III double digest is carried out on PJH4 resulting in linearization of the construct and clipping of a 12 $b_p$ segment which includes the ATG start codon of the PE coding sequence. Two complementary oligonucleotides are synthesized, annealed and ligated into the NdeI/Hind III splice site. The oligomers have the following nucleotide sequence: 1-5' TAT GCT GCA GGG TAC CAA GCT TAT GGC CGA AGA$^{3'}$ and II - 5' AGC TTC TTC GGC CAT AAG CTT GGT ACC CTG CAG CA3'. The modified PE insert has a sequence of MLQGTKLMAEE constructed at the N-terminus. This plasmid is designated PJH42.

The plasmid PJH42 is partially cut with Ava I. The linear form of DNA is isolated, completely digested with Hind III, and the resulting 5.1 Kb fragment isolated. S1 nuclease treatment is carried out

to allow blunt end ligation of the ends and the plasmid is recircularized and designated PJH43. This results in a PE with deletion of amino acids 4-252.

A 553 bp Sal I/Bam Hl fragment of plasmid PJH43 is cloned into M13 mp19. An oligonucleotide, 50 nucleotides in length with the structure 5' GGC GTC GCC GCT GTC CGC CGG GCC GTT GGC CGC GCC GGC CTC GTC GTT GC3', is synthesized and annealed to the single stranded M13 vector to facilitate (loop out) mutagenesis generating a deletion of amino acids 365-380 of the PE insert, resulting in the sequence:

....AGAANGPADSGDALL....
↑↑
364381

A 505 bp Sal I Bam Hl fragment is excised from the replicative form of the mutant DNA in M13 and ligated with a 3.7 Kb Sal l Bam Hl fragment of the plasmid PJH43. This new plasmid is designated PJH44.

A Bam Hl/EcoR l fragment of 460 nucleotides is excised from PJH44 and cloned into M13 mp19. This fragment contains the nucleotide sequence for three lysines that are mutated at the carboxy end of the coding sequence: lysines 590, 606 are mutated to glutamines and lysine 613 is mutated to an arginine. Oligo directed mutations are then carried out successively at each of the lysines with the following oligomers:

Lysine 590-5' GCT GAT CGC CTG TTC TTG GTC GGG GAT GCT GGA C 3'

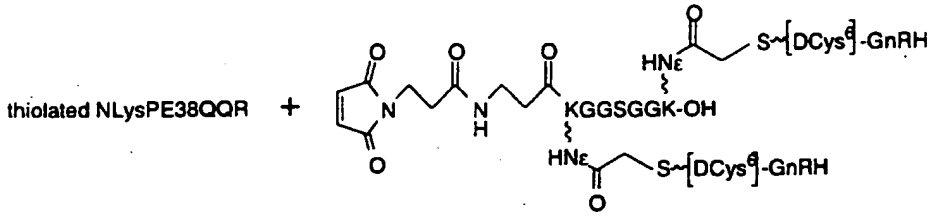
Lysine 606-5' GTC CTC GCG CGG CGG TTG GCC GGG CTG GCT G 3'

Lysine 613-5' CGG TCG CGG CAG TTA ACG CAG GTC CTC GCG CGG 3'

The Bam Hl EcoR l fragment is excised from the replicative form of the mutant DNA in M13 and ligated with a 3.4 Kb Bam Hl/EcoR

l fragment of the plasmid PJH44. The linearized plasmid is then recircularized, designated PJH45 and used for expression of the modified PE, identified as NLys PE38QQR, from a commercially available strain of E. coli, HB 101, available from Bethesda Research Laboratories.

## EXAMPLE 1
## [DCys$^6$]GNRH-LYS-GLY-GLY-SER-GLY-GLY-LYS-NLYS PE38QQR CONJUGATE




1) Preparation of Fmoc-β-Ala-Lys-Gly-Gly-Ser-Gly-Gly-Lys-OH (linear scaffold):

The linear scaffold was prepared on an ABI 431A peptide synthesizer (Fmoc chemistry) using pre-loaded Fmoc-Lys-WANG resin and single amino acid couplings. The peptide was cleaved from the resin TFA/thioanisole/ethenedithiol/anisole (90:5:3:2) and purified by gradient RP-HPLC.

2) Preparation of Fmoc-β-Ala-Lys($N_\varepsilon$HBrAc)Gly-Gly-Ser-Gly-Gly-Lys($N_\varepsilon$HBrAc)OH (bis-bromoacetylated linear scaffold):

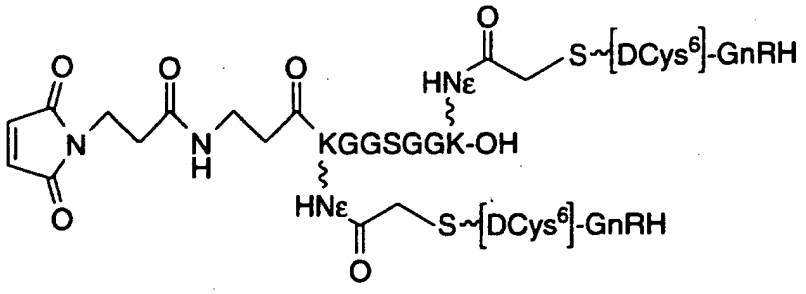
Bromoacetic anhydride was prepared by reaction of bromoacetic acid (31.5 mg, 0.226 mmol, 3.96 eq.) and DCC (23.4 mg, 0.113 mmol, 1.98 eq.) in dry (Aldrich Sure-Seal) dichloromethane (2 mL) at room temperature for 1 h. This mixture was filtered (sintered funnel, $N_2$ pressure) directly into a solution of the linear scaffold peptide from step 1 (50.0 mg, 0.057 mmol) that was dissolved in dry (4 Å sieves) degassed DMF (5 mL). The reaction was allowed to proceed at room temperature under $N_2$. Analysis of the reaction by RP-HPLC (Vydac $C_{18}$, 4.7X250 mm, 1.5 mL/min, 10->60% $CH_3CN$, 20 min) after 30 min indicated that all starting material had been consumed (ret. time for linear

scaffold =13.28 min) and a new product peak was observed (R.T.=16.48 min). After 45 min total reaction time, the mixture was concentrated *in vacuo* and the product purified by RP-HPLC (DeltaPak $C_{18}$, RCM 25X10, 10 ml/min, 25->55% $CH_3CN$, 30 min). The fractions containing the desired material were combined and lyophilized overnight, providing the bis-bromoacetylated linear scaffold as a white powder (31.2 mg, .027 mmol, 49%).

3) Preparation of Fmoc-β-Ala-Lys($N_\varepsilon HCOCH_2S$~[DCys$^6$]-GnRH)-Gly-Gly-Ser-Gly-Gly-Lys($N_\varepsilon HCOCH_2S$~[DCys$^6$]-GnRH)-OH (scaffold supported [DCys$^6$]-GnRH):

The bis-bromoacetylated linear scaffold from step 2 (31.2 mg, 0.027 mmol) was dissolved in degassed pH 8.0, 0.10 M phosphate buffer (5 mL) and acetonitrile (250 µL) was added. A pH meter indicated that the solution had a pH of 8.1. Next, [DCys$^6$]-GnRH (68.1 mg, 0.055 mmol, 2.04 eq.) was dissolved in water (2 mL) and a few drops of acetonitrile were added to clear the solution. The solution of [DCys$^6$]-GnRH was added dropwise (slow addition over 35 to 40 minutes) to the bis-bromoacetylated scaffold while maintaining the pH of the reaction mixture between pH 7.8 to 8.1 with the addition of dilute ammonium hydroxide. The final pH of the reaction mixture was 8.0. The reaction mixture was stirred at room temperature for 10 min then sonicated for 20 min after which time the mixture became cloudy. Stirring was again maintained at room temperature for a total of 2 h reaction time. RP-HPLC analysis of the reaction mixture (Vydac $C_{18}$, 4.7X250 mm, 1.5 mL/min, 10 to 60% $CH_3CN$, 20 min) indicated that all starting material was consumed and a new product peak was observed (R.T.=14.34. min). The reaction mixture was concentrated *in vacuo* and the remaining residue taken up in 6M guanidine hydrochloride and purified by RP-HPLC (DeltaPak $C_{18}$, RCM 25X10, 10 ml/min, 20 to 50% $CH_3CN$, 30 min). The fractions containing the desired material were combined and lyophilized overnight, providing the scaffold supported [DCys$^6$]-GnRH as a white powder (51 mg, .015 mmol, 49%).

4) Preparation of maleimidopropanoyl-β-Ala-Lys($N_\varepsilon$HCOCH$_2$S~[DCys$^6$]-GnRH)Gly-Gly-Ser-Gly-Gly-Lys($N_\varepsilon$HCOCH$_2$S~[DCys$^6$]-GnRH)-OH (maleimidated scaffold supported [DCys$^6$]-GnRH):




(a) Cleavage of Fmoc. The scaffold supported [DCys$^6$]-GnRH from step 3 (11.4 mg, 0.0033 mmol) was dissolved in 20% piperidine (Aldrich) in dry DMF (4Å sieves) (3 mL). The solution was stirred at room temperature for 45 min then concentrated *in vacuo*. The residue was taken up into ~15% aq. $CH_3CN$ and an aliquot was removed for RP-HPLC analysis. HPLC analysis showed approximately a 83:17 ratio of product to starting material (Vydac $C_{18}$, 4.7X250 mm, 1.5 mL/min, 10->60% $CH_3CN$, 20 min; RT=11.28 min deprotected peptide product, RT=14.72 min Fmoc-protected peptide starting material). The remaining material was lyophilized overnight affording the deprotected peptide as a white fluffy solid which was used directly in the maleimidation.

(b) Maleimidation of deprotected scaffold supported [DCys$^6$]-GnRH. The deprotected peptide (0.0033 mmol) was dissolved in dry (4Å sieves) degassed DMF (2.5 mL) and DIEA (10 μL) added. The mixture was stirred at room temperature and MPS (1.8 mg) was added in one portion. After 30 min an aliquot was removed for RP-HPLC analysis. HPLC analysis showed the appearance of two new products and a small amount of starting material (Vydac $C_{18}$, 4.7X250 mm, 1.5 mL/min, 10 to 60% $CH_3CN$, 20 min; RT=11.28 min deprotected peptide starting material, RT=12.13 min maleimidated peptide product). At T=45 min reaction time, the reaction mixture was quenched by the addition of

TFA (10 µL) and concentrated *in vacuo*. The remaining residue was taken up into 10% aq. $CH_3CN$ (0.1% TFA) and purified by RP-HPLC (DeltaPak $C_{18}$, RCM 25X10, 10 ml/min, 20 to 40% $CH_3CN$, 30 min). The fractions containing the desired material were combined and lyophilized overnight, providing the maleimidated scaffold supported [DCys$^6$]-GnRH as a white powder (6.9 mg, .0021 mmol, 62%). Electrospray MS showed that the product had the desired molecular weight (M+H=3348).

5) Thiolation of carrier protein NLys PE38QQR:

The protein (NLys PE38QQR, 1.28 mg/mL, 20 mL, 25.6 mg, 0.66 µmol) was placed in a 50 mL plastic sterile centrifuge tube and pH 11 borate buffer salts (832 mg, 41.6 mg/mL affords a 0.1 M solution of pH 11 borate buffer) were added and the mixture was capped and vortexed until all solids were in solution (5 min). EDTA (100 mg) and DTT (10 mg, 0.065 mmol) were added to the reaction mixture and the solution again vortexed until all solids were dissolved. The tube was transferred into an $N_2$-filled box and the cap replaced by a rubber septum. The tube was evacuated briefly and purged with $N_2$ (repeated 5X). N-acetylhomocysteine thiolactone (100 mg, 0.629 mmol) was added in one portion and the mixture vortexed until all solids were in solution and the tube re-evacuated and purged with $N_2$ (repeated 5X). The tube was capped and allowed to age in an $N_2$-filled box overnight (20 h) at room temperature. The reaction mixture was transferred to a dialysis bag and dialyzed against (a) 4L, 0.1 M pH 8.0 phosphate buffer with $N_2$ sparging (19 h); (b) 4L, 0.01 M pH 8.0 phosphate buffer containing 100 mg EDTA with $N_2$ sparging (8 h); and (c) 4L, 0.01 M pH 8.0 phosphate buffer containing 100 mg EDTA with $N_2$ sparging (20 h). The thiolated protein was transferred (in the $N_2$ box) to a 50 mL plastic centrifuge tube, approximately 24 mL volume. A 200 µL aliquot was removed for an Ellman's analysis ($OD_{412}$=0.155, 1.5 mL total volume) which revealed that the protein solution had a thiol titre of 0.083 µmol SH/mL solution.

6) Conjugation of Thiolated NLys PE38QQR and Maleimidated Scaffold Supported [DCys$^6$]-GnRH:

The maleimidated peptide of step 4 (5.0 mg 1.49 µmol) was dissolved in water (0.1% TFA) and placed in a sterile 50 mL plastic centrifuge tube and lyophilized overnight. The thiolated protein of step 5 (0.083 µmol SH/mL protein, 16.9 mL, 1.40 µmol) was added to the lyophilized peptide and the tube capped and vortexed briefly. The tube was sealed with parafilm and placed on a Clay-Adams nutator and tumbled overnight at 4 °C (17 h). The reaction mixture was transferred to a dialysis bag (Spectropor 2) and dialyzed (4 °C) *vs.* (a) 4L, 0.01 M pH 7.0 phosphate buffer (8 h); (b) 4L, pH 7.0 Dulbecco's phosphate buffered saline (PBS) (72 h), and (c) 4L, pH 7.0 Dulbecco's phosphate buffered saline (PBS) (24 h). There was no visible precipitate present. The conjugate was sterile filtered (sterile 50 mL Corning cup filter, 0.22 µM), providing approximately 15 mL of product. The conjugation product was characterized by CZE and SDS-PAGE gel electrophoresis. The concentration of the product was established (CZE) to be 1.1 mg/mL *vs.* known standards of TP40 (a chimeric protein containing transforming growth factor alpha at the N-terminus and a derivative of a 40 kDa segment (PE40deltacys) of Pseudomonas exotoxin) and NLys PE38QQR.

## EXAMPLE 2
## [DCys$^6$]GNRH-LYS-GLY-GLY-SER-GLY-GLY-LYS-OVALBUMIN CONJUGATE

1) Thiolation of Ovalbumin.

A thiolation mixture consisting of 86 mg ethylenediamine tetraacetic acid disodium salt (EDTA) and 15.4 mg of dithiothreitol (DTT) in 10 mL of pH 11 0.1M borate buffer was prepared. Ovalbumin (50.6 mg) was dissolved in this solution and then 49.6 mg of N-acetylhomocysteine thiolactone was added and this solution was filtered through a 0.22 m filter. The filtrate (9.2 mL) was degassed and after the air replaced by nitrogen was aged for 65 hr in a nitrogen box. This was then dialyzed against two 4L volumes of 0.01M pH 7.22 $PO_4$ buffer with

constant nitrogen sparging for 9 and 16 hr respectively affording a solution containing 486 nanomoles of thiol/ mL (Ellman assay). The solution was aged for 24 hr and then used in the next step.

2) Conjugation of thiolated ovalbumin and maleimidated scaffold supported [DCys$^6$]GnRH.

The pH of 2.0 mL of the thiolated ovalbumin solution (0.399 mmol SH / mL; 0.798 mmol SH total ) was adjusted to 7.94 with a saturated solution of diisopropylethylamine (DIEA) containing an equal amount of diisopropylethylamine hydrochloride. A coconjugation solution was prepared as follows: 0.83 mg of γ-maleimidobutyric acid as dissolved in 100 µL of water and 9 µL (408 nanomoles) of this solution was added to 100 µL of an aqueous solution containing the maleimidated scaffold supported [DCys6]GnRH of Example 1, step 4 (1.33 mg, 397 nanomoles). This coconjugation solution was added to the above thiolated ovalbumin solution and the mixture aged under $N_2$ for 3 hr at room temperature.

The conjugation mixture was then dialyzed in Spectapor 2 tubing vs. 4 L of water containing 1 mL of acetic acid (pH 3.64) for 15. 75 hr. A small precipitate was removed by centrifugation. The supernatant was then analyzed by HPSEC on a TSK 2000 column and found to be free of unconjugated scaffold. A control shows that 0.2 µg of the unconjugated scaffold would easily be detected. Amino acid analysis for β-alanine (a marker for the scaffold) and the constituent amino acids indicates that the conjugate solution contains 0.327 mg of scaffold/ml and 1.23 mg ovalbumin/mL.

## EXAMPLE 3
## PHOTOINACTIVATION OF THE CONJUGATE OF EXAMPLE 1

The conjugate of Example 1 (0.5 mL) was added to 9.5 mL of phosphate buffered saline (PBS) containing 4.16 mg of 8-azidoadenosine affording a solution which is 1.29 mM in conjugate and 1.35 mM in azidoadenosine. This solution was charged to a Pyrex

photoreactor with a cooling jacket through which ice water was pumped. It was then irradiated using a 450 W Hanovia lamp at a distance of 6 inches for 6 min. The solution was then dialyzed vs. 4 L of PBS 16 hr affording solution which had no low molecular weight materials as indicated by TSK 2000 HPSEC. The ADP ribosylating activity of this material was only 20% of the original as indicated by a wheat germ assay.

## EXAMPLE 4
## [DCys$^6$]GNRH-LYS-GLY-GLY-SER-GLY-GLY-LYS-UREASE CONJUGATE

1) Thiolation of urease:

EDTA (86.0 mg) and dithiothreitol (15.7) mg were charged to a 15 mL centrifuge tube dissolved in 10 mL of pH 11.0, 0.1M borate buffer. To this was added 181.6 mg urease (Sigma, 45% purity) and the solution was vortexed till all materials were dissolved. N-acetyl homocysteine thiolactone (181.4 mg) was then added to the solution. After vortexing, the solution was filtered through 0.22 μm syringe filters, de-gassed and the air replaced by nitrogen and allowed to react for 19 hr in an $N_2$ box. The solution was then dialyzed for 8 hr against 4 L 0.01M pH 7.3 phosphate buffer using Spectropor 2 (mwco 12-14000) tubing and then dialyzed again against a fresh reservoir of 0.01M pH 7.3 phosphate buffer for 16 hr.

2) Conjugation of thiolated urease and maleimidated scaffold supported [DCys$^6$]GnRH.

The thiolated urease solution ( 3.1 mL, 8.66 μmol total thiol titer based on Ellman assay) was treated with a saturated solution of DIEA (diisopropylethylamine) to raise the pH to 7.92 . The maleimidated scaffold supported [DCys$^6$]GnRH of Example 1, step 4 (1.42 mg, 0.423 μmol ) and 1.51 mg of γ-maleimidobutyric acid (8.24 μmol) were dissolved in approximately 500 μL of $H_2O$. This solution was added to the thiolated urease solution while vortexing and allowed to react for 2 hr. It was then dialyzed for 16 hr against 4L 0.01M pH 7.1

phosphate buffer using Spectropor 2 (MWCO 12-14000) tubing. Some precipitate formed in solution. The solution was centrifuged at low rpm in a clinical centrifuge and the supernatant was pipetted off from the precipitate. HPSEC using a TSK 2000 column showed an absence of any small molecules in the supernatant indicating that free scaffold had been removed. Amino acid analysis of the supernatant showed 4.4 mg urease per mL and a loading of 1.3 molecules of antigen per urease molecule. It also indicated that not all of the thiol titer had been consumed. A baseline thiol titer was established by an Ellman's assay. 10μL of an aqueous solution of 109.0 μg (0.595) γ-maleimido butyric acid was added to the supernatant while vortexing to cap the remaining thiols in the product. An Ellman assay showed no change in thiol titer. The supernatant was then dialyzed for 19.5 hr vs. 4 L PBS buffer using Spectropor 2 tubing affording the title product.

## EXAMPLE 5
## [DCys6]GNRH-LYS-GLY-GLY-SER-GLY-GLY-LYS-PHOTOINACTIVATED PSEUDOMONAS EXOTOXIN (HOLOTOXIN) CONJUGATE

1). Photoinactivation of PE holotoxin.

Pseudomonas Exotoxin A (10 mg, 0.152 mmol) (Sigma Chemical Co. ) was dissolved in 10 mL of water and to this was added 43 mg of 8-azidoadenosine (0.14 mmol). This did not completely dissolve and the small amount of precipitate was centrifuged and the supernatant was charged to a Pyrex photoreactor, cooled with ice water and irradiated with a 450 W Hanovia lamp for 8 min. The resultant solution was dialyzed for 17.5 hr against 4 L of PBS and then analyzed by TSK 2000 HPSEC which showed an absence of low molecular weight material (i.e. unreacted 8-azidoadenosine). The solution was also assayed for ADP ribosylating activity and found to have only 4% of the original level.

2) Thiolation of Photoinactivated PE holotoxin.

Borate buffer salt (43 mg [equal to 12.7 mmol/ mL of final solution] was added to 1 mL of water to which was added 10 mg of EDTA and 2.2 mg DTT. This thiolation medium was added to 9.5 mL of the photoinactivated PE toxin solution prepared in step 1. N-acetylhomocysteine thiolactone (11.3 mg) was added, the solution degassed and the air replaced with nitrogen and aged in a nitrogen box for 16 hr. It was then dialyzed against 4 L of PBS for 7.5 hr and a fresh 4 L of PBS for 65 hr at 4° C .The dialysis solutions were sparged with nitrogen throughout. An Ellman assay indicated 65 nanomoles of thiol / mL.

3) Conjugation of thiolated photoinactivated Pseudomonas exotoxin and maleimidated scaffold supported [DCys$^6$]GnRH.

The maleimidated scaffold supported [DCys$^6$]GnRH of Example 1, step 4 (1.88 mg, 450 nanomoles) was dissolved in 100 µL of water and 95 µL of this was added to 4 mL of the thiolated, photoinactivated PE holotoxin prepared in step 2. This was degassed and aged for 16 hr. At this time TSK 2000 HPSEC shows considerable low m.w. material remains. The solution was then dialyzed for 7 hr against 4L of PBS and then for 178 hr against a fresh 4 L of PBS at 4°C. An HPSEC assay shows no small molecules remain. An amino acid analysis indicates that there are 90 mg of scaffold / mL (β-alanine content) and 517 µg of PE toxin / mL.

## EXAMPLE 6
## [DCys$^6$]GNRH-LYS-GLY-GLY-SER-GLY-GLY-LYS-DIPHTHERIA TOXIN CONJUGATE

1) Thiolation of CRM197.

Borate buffer salt (pH 11, 42.6 mg; [3.4 mg / mL = 0.1M]) was added to 8.5 mL of water and to this was added 10 mg of EDTA and 2.3 mg of DTT (pH=9.82). This solution was used to dissolved 8 mg of CRM 197 ([Glu$^{52}$]-diphtheria toxin variant, Sigma Chemical Co.) . The pH of this solution was adjusted to 9.96 with 13 mL of 5N NaOH. Then

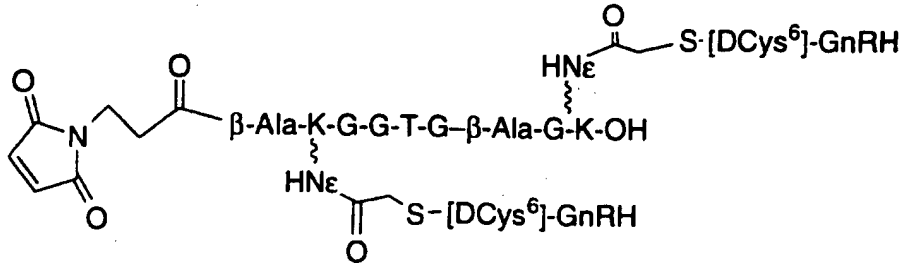
7.8 mg of N-acetylhomocysteine thiolactone was added and the mixture degassed and the air replaced by nitrogen. It was then aged in a nitrogen box for 18 hr. The reaction mixture was then dialyzed against 4 L of PBS for 9 hr and against a fresh 4 L of PBS for 20 hr in the cold room with constant nitrogen sparging. An Ellman assay indicates a titer of 117 nanomoles of SH / mL. After aging for 2 days this titer falls to 84 nanomoles / mL.

2) Conjugation of thiolated CRM197 and maleimidated scaffold supported [DCys$^6$]GnRH.

To 6 mL of the above thiolated CRM 197 solution was added 2.2 mg of the maleimidated scaffold supported [DCys$^6$]GnRH of Example 1, step 4 in approximately 600 µL of water. The solution was aged for 65 hr and centrifuged at low speed to remove a small amount of precipitate. The solution was dialyzed in Spectrapor 2 tubing against 4 L of PBS for 22 hr and in Spectrapor 7 against a fresh 4 L of PBS for 22 hr. and finally for 20 hr against a third 4L change of PBS. It was then assayed by TSK 2000 HPSEC and the low molecular weight peaks were absent. The resulting solution was analyzed by amino acid analysis and was found to contain 59 µg of scaffold / mL and 285 µg CRM197 / mL.

## EXAMPLE 7

Maleimidopropanoyl-βAla-Lys(N$_\varepsilon$HCOCH$_2$S~[DCys$^6$]-GnRH)-Gly-Gly-Thr-Gly-βAla-Gly-Lys(N$_\varepsilon$HCOCH$_2$S~[DCys$^6$]-GnRH)-OH



1) Preparation of linear scaffold-- Fmoc-βAla-Lys-Gly-Gly-Thr-Gly-βAla-Gly-Lys-OH:

The linear scaffold was prepared on an ABI 431A peptide synthesizer (Fmoc chemistry) using pre-loaded Fmoc-Lys-WANG resin (0.25 mmol) and single amino acid couplings. The peptide was cleaved from the resin (95:2.5:2.5 TFA/anisole/water) and purified by RP-HPLC (DeltaPak $C_{18}$, RCM 2-50X10, 45 mL/min, 20->35% $CH_3CN$, 30 min). The fractions containing the desired material were combined and lyophilized overnight, providing the peptide as a white powder (215 mg). ESI-MS (electrospray mass spectroscopy) M+H=968.

2) Bromoacetylated linear scaffold-- Fmoc-βAla-Lys(NεHBrAc)-Gly-Gly-Thr-Gly-βAla-Gly-Lys(NεHBrAc):

Bromoacetic anhydride was prepared by reaction of bromoacetic acid (86 mg, 0.62 mmol) and DCC (64 mg, 0.31 mmol) in dry (Aldrich Sure-Seal) dichloromethane (2 mL) at room temperature for 1 h. This mixture was filtered (sintered funnel, $N_2$ pressure) directly into a solution of the linear scaffold peptide (100 mg, 0.10 mmol) that was dissolved in dry (4 Å sieves) degassed DMF (5 mL) which contained DIEA (66 mg, 0.52 mmol). The reaction was allowed to proceed at room temperature under $N_2$. Analysis of the reaction by RP-HPLC (Vydac $C_{18}$, 4.7X250 mm, 1.5 mL/min, 10->60% $CH_3CN$, 20 min) after 45 min indicated that all starting material had been consumed (R.T.=13.26 min) and a new product peak was observed (R.T.=16.30 min). After 60 min total reaction time, the mixture was concentrated *in vacuo* and the product purified by RP-HPLC (DeltaPak $C_{18}$, RCM 2-50X10, 45 mL/min, 30->50% $CH_3CN$, 30 min). The fractions containing the desired material were combined and lyophilized overnight, providing the bis-bromoacetylated material as a white powder (36 mg, .030 mmol, 30%).

3) Protected Scaffold Supported [DCys$^6$]-GnRH--Fmoc-βAla-Lys(NεHCOCH$_2$S~[DCys$^6$]-GnRH)Lys-Gly-Gly-Thr-Gly-βAla-Gly-Lys(NεHCOCH$_2$S~[DCys$^6$]-GnRH)-OH:

The bis-bromoacetylated peptide (15 mg, 0.012 mmol) was dissolved in degassed pH 8.0, 0.10 M phosphate buffer (7 mL). A pH meter indicated that the solution had a pH of 8.0. Next, [DCys$^6$]-GnRH (31 mg, 0.025 mmol) was dissolved in water (2 mL). The solution of [DCys$^6$]-GnRH was added dropwise (*via* syringe pump, 1 hr) to the activated scaffold. The heterogeneous reaction mixture was stirred at room temperature for an additional 30 min. The reaction mixture was concentrated *in vacuo* and the remaining residue taken up in 6M guanidine hydrochloride and purified by RP-HPLC (DeltaPak C$_{18}$, RCM 25X10, 10 mL/min, 25->45% CH$_3$CN, 30 min). The fractions containing the desired material were combined and lyophilized overnight, providing the bis-[DCys$^6$]-GnRH scaffold supported peptide as a white powder (27 mg, 65%). ESI-MS (electrospray mass spectroscopy) M+H=3505.

4) Maleimidated Scaffold Supported [DCys$^6$]-GnRH -- Maleimidopropanoyl-βAla-Lys(NεHCOCH$_2$S~[DCys$^6$]-GnRH)-Gly-Gly-Thr-Gly-βAla-Gly-Lys(NεHCOCH$_2$S~[DCys$^6$]-GnRH)-OH:

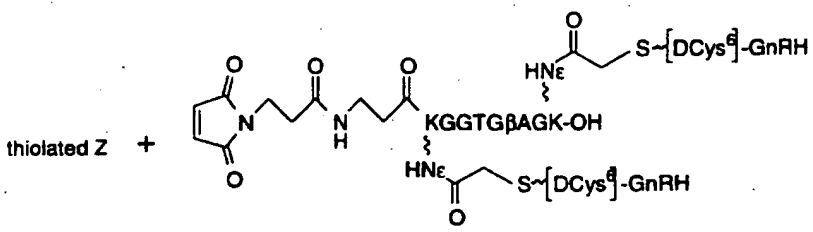
The peptide (27 mg, 0.008 mmol) was dissolved in 20% piperidine (Aldrich) in dry DMF (4Å sieves) (20 mL). The solution was stirred at room temperature for 1 hr then concentrated *in vacuo*. Analysis of the reaction by RP-HPLC (Vydac C$_{18}$, 4.7X250 mm, 1.5 mL/min, 10->60% CH$_3$CN, 20 min) indicated that a new product peak was observed (R.T.=11.68 min). The product was purified by RP-HPLC (DeltaPak C$_{18}$, RCM 25X10, 10 mL/min, 15->35% CH$_3$CN, 30 min). The fractions containing the desired material were combined and lyophilized overnight, providing the deprotected peptide as a white powder (18 mg, 70%).

The deprotected peptide (0.0054 mmol) was dissolved in dry (4Å sieves) degassed DMF (5 mL) and DIEA (5 µL) added. The mixture was stirred at room temperature and MPS (3 mg, 0.011 mmol) added in one portion. At T=1 hr reaction time, the mixture was quenched by the addition of TFA (10 µL) and concentrated *in vacuo*. The remaining residue was taken up into $CH_3COOH$/10% aq. $CH_3CN$ (0.1% TFA) and purified by RP-HPLC (DeltaPak $C_{18}$, RCM 25X10, 10 mL/min, 15->35% $CH_3CN$, 30 min). The fractions containing the desired material were combined and lyophilized overnight, providing the maleimido bis-[$DCys^6$]-GnRH scaffold supported peptide as a white powder (11 mg, 57%). Electrospray MS showed that the product had the desired molecular weight (M+H=3434).

## EXAMPLE 8

## Conjugates of [$DCys^6$]GnRH scaffold of Example 7 ([DCys6]GnRH-LysGlyGlyThrGlyβAlaGlyLys)

Conjugates are made by following the general procedures of Examples 1 - 6, and using the GnRH scaffold of Example 7.

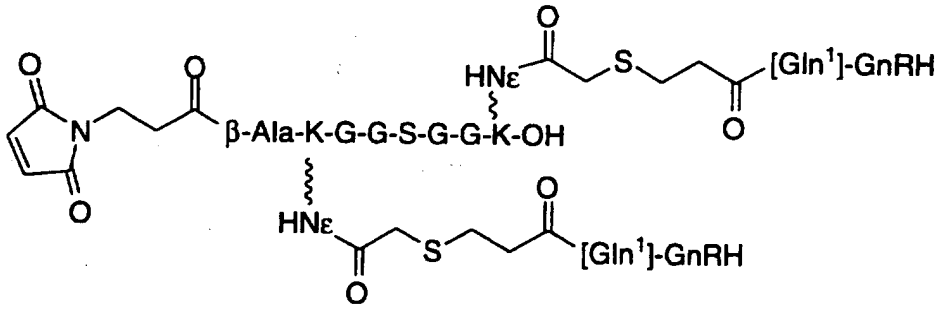



βA is βAla.

a. Z = NLysPE38QQR
b. Z = ovalbumin
c. Z = NLysPE38QQR (photoinactivated form of a)
d. Z = urease
e. Z = photoinactivated PE holotoxin
f. Z = diphtheria toxin variant

## EXAMPLE 9

Maleimidopropanoyl-βAla-Lys(NεHCOCH$_2$S~[(3-mercaptopropanoyl)-Gln$^1$]-GnRH)-Gly-Gly-Ser-Gly-Gly-Lys(NεHCOCH$_2$S~[(3-mercaptopropanoyl)-Gln$^1$]-GnRH)-OH




1) Protected Scaffold Supported [(3-mercaptopropanoyl)-Gln$^1$]-GnRH--Fmoc-βAla-Lys(NεHCOCH$_2$S~[(3-mercaptopropanoyl)-Gln$^1$]-GnRH)Gly-Gly-Ser-Gly-Gly-Lys(NεHCOCH$_2$S~[(3-mercaptopropanoyl)-Gln$^1$]-GnRH)-OH:

The bis-bromoacetylated peptide of Example 1, step 2 (25 mg, 0.022 mmol) was dissolved in degassed pH 8.0, 0.10 M phosphate buffer (15 mL). A pH meter indicated that the solution had a pH of 8.0. Next, [(3-mercaptopropanoyl)-Gln$^1$]-GnRH (57 mg, 0.045 mmol) was dissolved in water (2 mL). The solution of [(3-mercaptopropanoyl)-Gln$^1$]-GnRH was added dropwise (*via* syringe pump, 1 hr) to the activated scaffold. The heterogeneous reaction mixture was stirred at room temperature for an additional 30 min. The reaction mixture was concentrated *in vacuo* and the remaining residue taken up in 6M guanidine hydrochloride and purified by RP-HPLC (DeltaPak C$_{18}$, RCM 25X10, 10 mL/min, 25->50% CH$_3$CN, 30 min). The fractions containing the desired material were combined and lyophilized overnight, providing the bis-[(3-mercaptopropanoyl)-Gln$^1$]-GnRH scaffold supported peptide as a white powder. ESI-MS (electrospray mass spectroscopy) M+H=3538.

2) Maleimidated Scaffold Supported[(3-mercaptopropanoyl)-Gln$^1$]-GnRH -- Maleimidopropanoyl-βAla-Lys($N_\varepsilon$HCOCH$_2$S~[(3-mercaptopropanoyl)-Gln$^1$]-GnRH)-Gly-Gly-Ser-Gly-Gly-Lys($N_\varepsilon$HCOCH$_2$S~[(3-mercaptopropanoyl)-Gln$^1$]-GnRH)-OH:

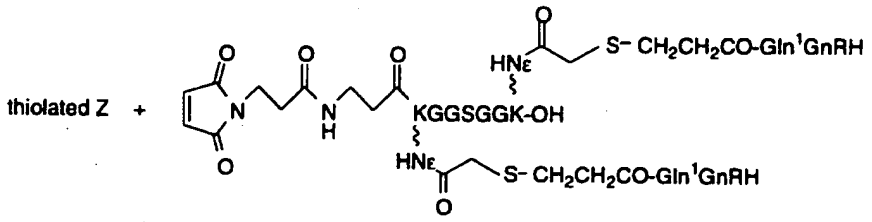
The peptide (32 mg, 0.009 mmol) was dissolved in 20% piperidine (Aldrich) in dry DMF (4Å sieves) (20 mL). The solution was stirred at room temperature for 1 hr then concentrated *in vacuo*. Analysis of the reaction by RP-HPLC (Vydac $C_{18}$, 4.7X250 mm, 1.5 mL/min, 10->60% $CH_3CN$, 20 min) indicated that a new product peak was observed (R.T.=11.95 min). The product was purified by RP-HPLC (DeltaPak $C_{18}$, RCM 25X10, 10 mL/min, 15->35% $CH_3CN$, 30 min). The fractions containing the desired material were combined and lyophilized overnight, providing the deprotected peptide as a white powder (19 mg, 61%).

The deprotected peptide (0.0056 mmol) was dissolved in dry (4Å sieves) degassed DMF (5 mL) and DIEA (5 μL) added. The mixture was stirred at room temperature and MPS (3 mg, 0.011 mmol) added in one portion. At T=1 hr reaction time, the mixture was quenched by the addition of TFA (20 μL) and concentrated *in vacuo*. The remaining residue was taken up into $CH_3COOH$/10% aq. $CH_3CN$ (0.1% TFA) and purified by RP-HPLC (DeltaPak $C_{18}$, RCM 25X10, 10 mL/min, 15->35% $CH_3CN$, 30 min). The fractions containing the desired material were combined and lyophilized overnight, providing the maleimido bis-[(3-mercaptopropanoyl)-Gln$^1$]-GnRH scaffold supported peptide as a white powder (11 mg, 57%). Electrospray MS showed that the product had the desired molecular weight (M+H=3468).

## EXAMPLE 10

## Conjugates of [Gln$^1$]GnRH scaffold of Example 9 ([Gln$^1$]GnRH-Lys-Gly-Gly-Ser-Gly-Gly-Lys)

Conjugates are made by following the general procedures of Examples 1 - 6, and using the GnRH scaffold of Example 9.




a. Z = NLysPE38QQR
b. Z = ovalbumin
c. Z = NLysPE38QQR (photoinactivated form of a)
d. Z = urease
e. Z = photoinactivated PE holotoxin
f. Z = diphtheria toxin variant

## EXAMPLE 11

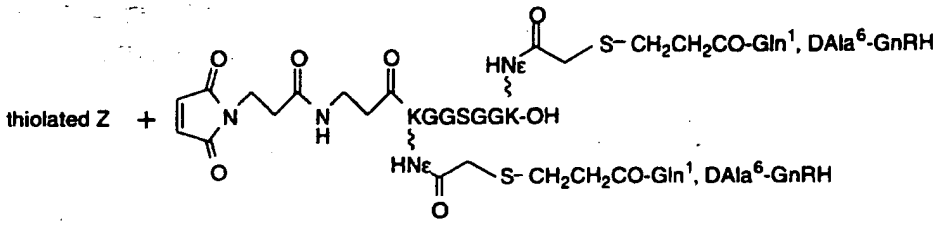
## Maleimidopropanoyl-βAla-Lys(NεHCOCH2S~[(3-mercaptopropanoyl)-Gln1, D-Ala6]-GnRH)-Gly-Gly-Ser-Gly-Gly-Lys(NεHCOCH2S~[(3-mercaptopropanoyl)-Gln1, D-Ala6]-GnRH)-OH

The title compound is prepared following the general procedure described in Example 9, and using [(3-mercaptopropanoyl) Gln1, D-Ala6]GnRH of Preparation 3.

## EXAMPLE 12

## Conjugates of [Gln1, D-Ala6]GnRH scaffold of Exmaple 11

Conjugates are made by following the general procedures of Examples 1 - 6, and using the GnRH scaffold of Example 11.




a. Z = NLysPE38QQR
b. Z = ovalbumin
c. Z = NLysPE38QQR (photoinactivated form of a)
d. Z = urease
e. Z = photoinactivated PE holotoxin
f. Z = diphtheria toxin variant

## EXAMPLE 13

### Vaccine Preparation and Efficacy Screening

The vaccines were prepared by combining the peptide solutions with 0.9% sodium chloride injection USP (Baxter, Lot C255075) and Incomplete Freund's Adjuvant (Sigma, F 5506, Lot 062H-8802) in the proportions listed below in a 20 cc, glass, luer-lok syringe (Popper & Sons). Homogenization was achieved by passing the mixture between two 20 cc glass syringes through a 20 gauge, double hubbed, homogenization needle (Popper & Sons) until stiff.

| Vaccine No. | Antigen (Ex.1 in mg/ml)* | Saline | IFA | Final Ag Conc. |
|---|---|---|---|---|
| 1 | 3 ml (1.17) | 4 ml | 7 ml | 250 ug/ml |
| 2 | 6 ml (0.117) | 1 ml | 7 ml | 50 ug/ml |
| 3 | 0.6 ml (0.117) | 6.4 ml | 7 ml | 5 ug/ml |
| 4 | 6 ml (0.00117) | 1 ml | 7 ml | 0.5 ug/ml |

* the numbers in parenthesis represent the concentrations of the conjugate of Example 1.

In addition, an alum adjuvanted vaccine was also prepared by mixing 6 ml of immunogconjugate of Example 1 (0.117 mg/ml in saline), 1 ml saline and 7 ml Alhydrogel 1.3% (Superfos Biosector a/s, Batch 1983) to yield antigen final concentration of 50 ug/ml (vaccine No. 5).

Vaccination:

Twenty-seven 10 week-old, castrated male pigs were divided into six groups: one group of 5 animals for each of the above vaccine preparations plus a group of 2 control animals. Pretreatment blood samples (10 ml) were collected by jugular venopuncture and each pig was vaccinated with two mls of freshly prepared vaccine (1 ml intramuscularly on each side of the neck). Four weeks later, blood (10 ml) was again collected from all animals and they were revaccinated with freshly prepared vaccine as previously described. Two weeks later, all animals were bled three times at approximately 1 hour intervals. All blood samples were centrifuged following collection and serum was frozen at -20 C until all of the bleeding were completed so that all serum could be assayed in the same antibody titer and LH analyses.

Antibody Titer Analysis:

PBS-BSA was prepared by dissolving one packet of BupH modified Dulbecco's phosphate buffered saline mix (Pierce, No. 28374, Lot 920521084) in 400 ml of deionized water and adding 2 gm of bovine albumin fraction V (Gibco No. 810-1018IL, Lot 76P9623) and 5 ml of a 1% w/v thimerosal solution (Sigma, T-5125, Lot 23H0526). Once the BSA was dissolved, deionized water was added to a final volume of 500 ml.

Dextran-coated charcoal suspension was prepared by washing 2.5 g of charcoal (Sigma, C-5385, Lot 102H0336 )with deionized water multiple times to remove the fines. PBS was prepared by dissolving two packet of BupH Modified Dulbecco's Phosphate Buffered Saline mix (Pierce, No. 28374, Lot 920521084) in 1000 ml of deionized water. 0.25g of dextran, 70,000 mw (Sigma, D1390, Lot 122H0349) was dissolved in 500 ml of PBS. The washed charcoal was added to this

solution. 10 ml of 1% w/v thimerosal solution (Sigma, T-5125, Lot 23H0526) plus an additional 500 ml of PBS were added and the charcoal suspension was stirred for 3 days at 4 C.

All serum samples were thawed. A 100 fold dilution of serum was prepared by adding 5 ul of serum to 495 ul of PBS-BSA. A 1000 fold dilution of serum was prepared by adding 50 ul of the 100 fold dilution to 450 ul of PBS-BSA. 10,000 and 100,000 fold dilutions were prepared similarly. Fifty ul aliquots of all serum dilutions of were added to duplicate 12x75 borosilicate glass tubes (Fisher) (two tubes per dilution). To each tube were added 50 ul of PBS-BSA containing 400,000 cpm/ml of $^{125}$I labeled-GnRH (NEN, NEX-163, Lot CF91640). Tubes were incubated overnight at 4 C. Then 100 ul of dextran coated charcoal suspension was added and tubes were mixed at room temperature for 15 minutes. Tubes were then placed in a Sorval RC-3B centrifuge and spun at 2500 rpm in an H6000 A rotor. A 100 ul aliquot of the supernatant was collected and the radioactivity was quantified in a Packard AutoGamma 800 gamma counter. Results were expressed as percentage binding of the total input radioactivity (determined by adding 50 ul of the $^{125}$I labeled GnRH solution to 150 ul of PBS-BSA, centrifuging and counting 100 ul).

The antibody titer results were as follows:
Values are expresses as the average percentage of input $^{125}$I-labeled peptide which was bound at a 1/1000 dilution of serum except for pretreatment which is neat serum.

| Vaccine No. | Pretreatment | 4 Weeks | 6 Weeks |
|---|---|---|---|
| 1 | 1.4% | 15.0% | 52.7% |
| 2 | 1.2% | 8.2% | 39.2% |
| 3 | 1.4% | 5.9% | 37.1% |
| 4 | 1.0% | 4.1% | 12.3% |
| 5 | 0.9% | 0.5% | 8.0% |
| Control | 0.5% | -0.3% | -0.4% |

Serum LH Determination

Serum samples were submitted to the USDA lab at Athens, GA under the supervision of Dr. George Rampacek for serum LH quantitation by radioimmunoassay. The assay is a standard radioimmunoassay using $^{125}I$ labelled porcine LH and anti-bovine LH antisera which recognize porcine LH. This assay is described by R. Kraeling et al in *J. Anim. Sci.* (1982) 54:1212. The results below are the means for each group. Week 6 is the pooled mean for the three bleedings. The results are presented in ng of LH/ml. 0.15 ng LH/ml srumis the lower limit of the assay so all values which fell below the levell of detection were assigned the value of 0.15 ng/ml.

| Vaccine No. | Pretreatment | 4 Weeks | 6 Weeks |
|---|---|---|---|
| 1 | 1.85 | 0.15 | 0.15 |
| 2 | 1.46 | 0.44 | 0.15 |
| 3 | 1.41 | 0.52 | 0.15 |
| 4 | 0.87 | 0.85 | 0.16 |
| 5 | 0.82 | 0.72 | 0.24 |
| Control | 1.50 | 1.22 | 0.96 |

EXAMPLE 14

Vaccine formulation with STP

1. Preparation of STP (5% Squalane: 0.2% Tween® 80: 2.5 % Pluronic® L121 in phosphate buffered saline [PBS] ) :

Squalane (500 mg), Pluronic® 121 (250 mg; a block copolymer of polyethylene oxide and polypropylene oxide (BASF corp.) and Tween® 80 (20 mg) are weighed into a 15 mL Dounce tissue homogenizer tube. This is then covered with 9.25 mL of PBS (pH 7.4) and the resultant mixture homogenized with about ten strokes . The solution is then transferred to a vial and a small magnetic stir bar added.

About 3 mL of this mixture is transferred into cylinder of an Avestin Emulsiflex® microfluidizer. and the exit tube of the Emulsiflex is positioned so that it is submerged below the surface of the remaining liquid in the vial The vial is cooled in an ice bath, and twenty passes of the Emulsiflex are effected while the liquid is magnetically stirred. In this manner 9.5 mL of STP are obtained.

2. Formulation of the conjugate of Example 1 in STP 130 µL of a solution of the conjugate of Example 1 (1µg /µL of total immunogen = 130 µg total) is added with magnetic stirring to 6.4 mL of the above STP emulsion and the resultant mixture subjected to 10 passes in the Emulsiflex® unit using the same procedure as in the preparation of STP. This affords about 6 mL of the conjugate formulated in STP at a concentration of 20 µg/ mL. The same product can be obtained by adding the requisite amount of the conjugate (in PBS) to the squalane:Tween® 80:Pluronic® L121 mixture in the Dounce homogenizer in Procedure 1, and subjecting the four part mixture to 20 passes in the microfluidizer.

3. Formulation of the conjugate of Example 1 in STP with Dehydroepiandrosterone (DHEA): : 130 µL of solution of the conjugate of Example 1 (1µg /µL of total immunogen = 130 µg total) is added with magnetic stirring to 6.2 mL of the STP emulsion prepared in section 1. A solution of DHEA (10 µg/µL) in ethanol is prepared and 195 µL of this solution (1.95 mg) is added to the stirred conjugate solution in STP. This mixture is then subjected to 10 passes through the Emulsiflex instrument, to afford about 6 mL of conjugate in STP with 300 µg/mlDHEA.

The general procedure of Steps 2 and 3 was followed to provide vaccines in STP or STP+DHEA containing the conjugate of Example 2, [DLys$^6$]GnRH-NLysPE38QQR (see WO93/15751), or [DLys$^6$]GnRH-ovalbumin (supra, Preparation 4).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: LOMBARDO, VICTORIA K.
MARBURG, STEPHEN
TOLMAN, RICHARD L.

(ii) TITLE OF INVENTION: CONJUGATES OF GONADOTROPIN RELEASING HORMONE

(iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: RAHWAY
(D) STATE: NEW JERSEY
(E) COUNTRY: USA
(F) ZIP: 07065

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: YANG, MOLLIE M.
(B) REGISTRATION NUMBER: 32,718
(C) REFERENCE/DOCKET NUMBER: 19444

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 908-594-6343
(B) TELEFAX: 908-594-4720

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Xaa His Trp Ser Tyr Xaa Xaa Arg Xaa Xaa
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Xaa His Trp Ser Tyr Gly Leu Arg Pro Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Xaa His Trp Ser Tyr Xaa Leu Arg Pro Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Xaa Lys Gly Gly Ser Ser Gly Gly Lys
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Xaa Lys Gly Gly Thr Gly Xaa Gly Lys
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 356 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Leu Gln Gly Thr Lys Leu Met Ala Glu Glu Gly Gly Ser Leu Ala
1 5 10 15

Ala Leu Thr Ala His Gln Ala Cys His Leu Pro Leu Glu Thr Phe Thr
20 25 30

Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Cys Gly Tyr
35 40 45

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Pro Val Gln Arg Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser Trp
    50                  55                  60

Asn Gln Val Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro Gly Ser
65                  70                  75                  80

Gly Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala Arg
                85                  90                  95

Leu Ala Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg Gln
            100                 105                 110

Gly Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Gly Pro Ala Asp Ser
        115                 120                 125

Gly Asp Ala Leu Leu Glu Arg Asn Tyr Pro Thr Gly Ala Glu Phe Leu
    130                 135                 140

Gly Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly Thr Gln Asn Trp
145                 150                 155                 160

Thr Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg Gly
                165                 170                 175

Tyr Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser
            180                 185                 190

Ile Val Phe Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile
        195                 200                 205

Trp Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly Tyr
    210                 215                 220

Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn Gly Ala
225                 230                 235                 240

Leu Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr Arg
                245                 250                 255

Thr Ser Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu Arg
            260                 265                 270

Leu Ile Gly His Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr Gly Pro
        275                 280                 285

Glu Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu Ala
    290                 295                 300

Glu Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg Asn
305                 310                 315                 320

Val Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Gln Glu Gln Ala
                325                 330                 335

Ile Ser Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly Gln Pro Pro Arg
```

340 345 350

Glu Asp Leu Arg
355

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Xaa His Trp Ser Tyr Xaa Leu Arg Pro Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Gln His Trp Ser Tyr Gly Leu Arg Pro Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gln His Trp Ser Tyr Xaa Leu Arg Pro Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gly Gly Ser Gly Gly
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

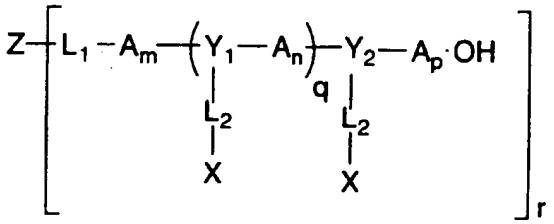
(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Gly Gly Thr Gly Xaa Gly
1 5

WHAT IS CLAIMED IS:

1. A conjugate of formula I:



I

wherein

A is independently an amino acid selected from Gly, Ser, Thr, β-Ala and Ala, with the proviso that at least one A is Ser or Thr;

$L_1$ is a linker optionally attached to an internal marker;

$L_2$ is independently a linker;

X is a GnRH modified at positions 1, 6 or 10 for linker enablement;

$Y_1$ and $Y_2$ are independently Lys or Orn;

Z is an immunogenic carrier protein;

m is 0 to 3;

n is 1 to 10;

p is 0 to 1;

q is 1 or 2;

r is 1 to 10.

2. A conjugate of Claim 1 wherein Z is selected from the group consisting of Pseudomonas exotoxin or a variant thereof, and ovalbumin.

3. A conjugate of Claim 1 wherein X is [SEQUENCE ID NO: 1]

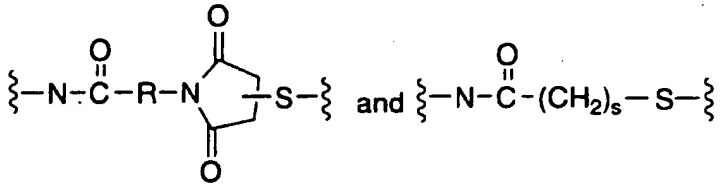
$(B)_pQ$-His-Trp-Ser-Tyr-W-T-Arg-U-V

wherein

| | |
|---|---|
| B is | a thiol containing linker of the formula HS-(CH2)n-CO-; |
| p is | 0 to 1; |
| n is | 1 to 10; |
| Q is | pGlu or Gln; and |
| W is | a D- or L- amino acid selected from glycine, alanine, cysteine, homocysteine, ornithine or lysine; |
| T is | Leu or Nle; |
| U is | Pro or 4-hydroxy-Pro; and |
| V is | Gly-$NH_2$, D-Ala-$NH_2$, NH-Et, NH-Pr or Arg-Gly-$NH_2$; |

with the proviso that the GnRH is linked to $L_2$ via an amino or a sulfhydryl group on Q or W.

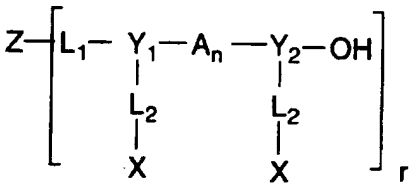
4. A conjugate of Claim 1 wherein $L_1$ and $L_2$ are selected from




wherein:

| | |
|---|---|
| R is | $C_1$-$C_5$ alkylene, phenyl or $C_5$-$C_6$ cycloalkylene; |
| s is | 1 or 2; |
| $L_1$ is | attached to β-Ala; and |
| Z is | selected from Pseudomonas exotoxin, or a variant thereof, and ovalbumin. |

5. A conjugate of Claim 1 having the formula



wherein

n is 3 to 8;

r is 1 to 3; and

A, X, $Y_1$, $Y_2$, Z, $L_1$ and $L_2$ are as defined in Claim 1.

6. A conjugate of Claim 5 wherein Z is selected from Pseudomonas exotoxin Lys PE-38QQR, NLys PE-38QQR and ovalbumin.

7. A conjugate of Claim 5 wherein X is [SEQUENCE ID NO: 3]

$(B)_p$Q-His-Trp-Ser-Tyr-W-Leu-Arg-Pro-Gly-$NH_2$

wherein

B is a thiol containing linker of the formula HS-(CH2)n-CO-;

p is 0 or 1;

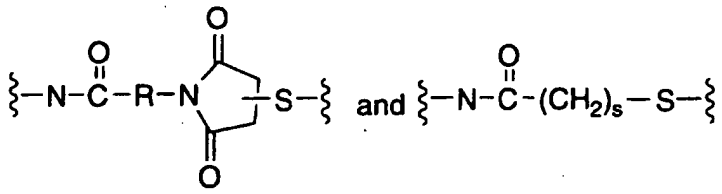
n is 1 to 10;

Q is pGlu or Gln; and

W is a D- or L- amino acid selected from glycine, alanine, cysteine, homocysteine, ornithine or lysine;

with the proviso that at least one of Q or W has a free amino or sulfhydryl group though which GnRH is linked to $L_2$.

8. A conjugate of Claim 5 wherein $L_1$ and $L_2$ are selected from



wherein:

R is $C_1$-$C_5$ alkylene, phenyl or $C_5$-$C_6$ cycloalkylene;

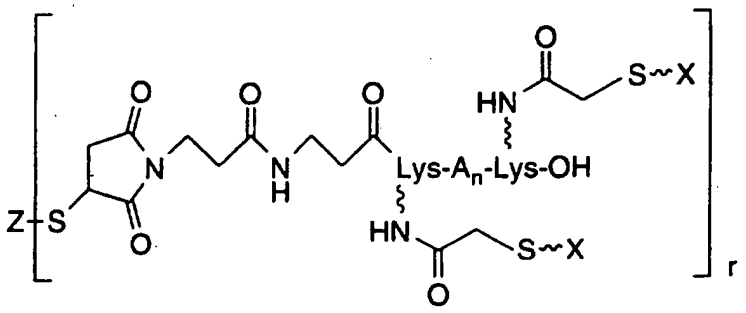
s is 1 or 2;

$L_1$ is attached to β-Ala; and

Z is selected from Pseudomonas exotoxin, or a variant thereof, and ovalbumin.

9. A conjugate of Claim 5 wherein n is 4 to 7; one of A is Thr or Ser, and the others are selected from Gly, Ala and β-Ala..

10. A conjugate of Claim 5 having the formula:



wherein

one of A is Ser or Thr, and the others are selected from Gly, Ala and β-Ala;

X is a GnRH having a free sulfhydryl group;

Z is an immunogenic carrier protein;

n is 5 or 6; and

r is 1 to 3.

11. A conjugate of Claim 10 wherein $A_n$ is selected from [SEQUENCE ID NO: 10] Gly-Gly-Ser-Gly-Gly and [SEQUENCE ID NO: 11] Gly-Gly-Thr-Gly-βAla-Gly, and X is selected from [DCys$^6$]GnRH and HSCH$_2$CH$_2$CO-[Gln$^1$]GnRH.

12. A conjugate of Claim 10 wherein $A_n$ is [SEQUENCE ID NO: 10] Gly-Gly-Ser-Gly-Gly, and X is selected from [DCys$^6$]GnRH and HSCH$_2$CH$_2$CO-[Gln$^1$]GnRH.

13. A method for sterilizing an animal comprising administering to said animal a conjugate of Claim 1 in an amount effective to elicit anti-GnRH antibodies.

14. A vaccine composition comprising an immune response stimulating effective amount of a GnRH-conjugate in an oil-in-water emulsion vehicle which comprises:

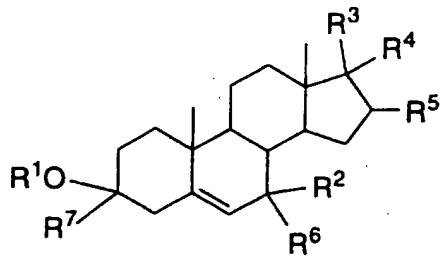
(a) a metabolizable oil;

(b) a non-ionic surfactant; and

(c) an emulsifier.

15. A composition of Claim 14 wherein said non-ionic surfactant is a polyoxypropylene-polyethylene block polymer.

16. A composition of Claim 14 further comprising an immunopotentiating amount of an immune response enhancer having the formula:




1

wherein

$R^1$ is H, $C_{2-8}$ alkenyl, $C_{1-8}$ alkyl, benzyl, phenyl or $COR^4$, wherein $R^4$ is H, $C_{1-8}$ alkyl, $C_{2-8}$ alkenyl, benzyl or phenyl wherein the phenyl moiety may have up to three substituents selected from the group consisting of hydroxy, carboxy of 1-4 carbon atoms, halo, $C_{1-4}$ alkoxy, $C_{1-4}$ alky, and $C_{2-4}$alkenyl, $SO_3M$ or $PO_3M$, wherein M is H or sodium or potassium;

$R^2$ is H or $OR^1$;

$R^3$ is $OR^1$ or $R^3$ and R4 together form an oxo;

$R^4$, $R^5$, $R^6$, and $R^7$ are independently H or methyl;

with the proviso that when $R^3$ and R4 together form an oxo, $R^5$, $R^6$, $R^7$ and $R^2$ are each H; and that when $R^2$ is H, $R^4$, $R^5$, $R^6$ and $R^7$ are each hydrogen, and $R^3$ is $OR^1$.

17. A composition of Claim 14 comprising an immune response stimulating effective amount of a GnRH conjugate in an oil-in-water emulsion vehicle which comprises:

(a) squalane;

(b) polyoxypropylene-polyoxyethylene block polymer wherein POP has a molecular weight of 4000 and POE in an amount of 10%; and

(c) polyoxyethylene 20 sorbitan monooleate.

18. A composition of Claim 17 further comprising an immunopotentiating amount of an immune response enhancer having the formula 1, wherein $R^1$, $R^2$, $R^5$, $R^6$, $R^7$ are each H, and $R^3$ and $R^4$ together form an oxo.

19. A method for sterilizing an animal comprising administering to said animal a vaccine composition of Claim 14 in an amount effective to elicit anti-GnRH antibodies.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/16950

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 38/00, 38/24, 39/00, 39/385; C07K 1/00, 7/06, 14/59, 17/06
US CL :424/184.1, 193.1, 194.1, 195.11, 197.11; 530/333, 399
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 193.1, 194.1, 195.11, 197.11; 530/333, 399

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y, P | US 5,492,893 A (NETT et al) 20 February 1996, see entire document. | 1-19 |
| Y,P | US 5,488,036 A (NETT et al) 30 January 1996, see entire document. | 1-19 |
| Y | WO 93/15751 A (MERCK & CO., INC.) 19 August 1993, see entire document. | 1-19 |
| Y | GB 2,282,812 A2 (MERCK & CO., INC.) 19 April 1995, see entire document. | 1-19 |
| Y | WO 90/09799 A (COLORADO STATE UNIVERSITY RESEARCH FOUNDATION) 07 September 1990, see entire document. | 1-19 |
| Y,P | US 5,540,919 A (DAYNES et al) 30 July 1996, see entire | 14-19 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion of the international search<br>24 JANUARY 1997 | Date of mailing of the international search report<br>21 FEB 1997 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>PATRICIA A. DUFFY<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/16950

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US 4933179 A (ALLISON et al) 12 June 1990, see entire document. | 14-19 |
| Y | US 4806350 A (GERBER, JAY D.) 21 February 1989, see entire document. | 14-19 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/16950

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/16950

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE, MEDLINE, DIALOG ONSEARCH - MEDICINE, BIOSYS, EMBASE, DERWENT WPI, JAPIO.
search terms: GNRH, gonadotropin releasing hormone, vaccine, contracept?, conjugat?, link?, attach?, adjuvant, steroid, enhanc?, oil, emulsifier, detergent, surfactant, POE, POP, squalane, sorbitan, polyoxypropylene-polyoxycthylene, serilization, castration, structure search.